Comparative Study of Inhibitory Potential of Dietary Phytochemicals Against Quorum Sensing Activity of and Biofilm Formation by *Chromobacterium violaceum* 12472, and Swimming and Swarming Behaviour of *Pseudomonas aeruginosa* PAO1

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SUMMARY

Quorum sensing (QS) and biofilm formation are important mechanisms related to antibiotic resistance of many pathogens. Alternative treatments are needed to prevent recurrent or chronic infections caused by multi-resistant pathogens. Therefore, the aim of this study is to investigate and compare the inhibitory potential of the dietary phytochemicals: curcumin, quercetin, apigenin, pyrogallol, gallic acid and luteolin against QS of and biofilm formation by *Chromobacterium violaceum* ATCC 12472 and the swimming and swarming abilities of *Pseudomonas aeruginosa* PAO1. Anti-QS potential of the phytochemicals was evaluated qualitatively and quantitatively using *C. violaceum via* the disk diffusion assay based on violacein pigment inhibition at the subminimal inhibitory concentrations ranging from 46.87 to 750 µg/mL. The results of anti-QS and antibiofilm activities on *C. violaceum* demonstrated that all the phytochemicals except pyrogallol and gallic acid inhibited violacein production (from (11.0±0.1) to (88.2±0.1) %) in a concentration-dependent manner. In addition, the biofilm formation was also significantly inhibited ($p<0.05$) in the presence of all the phytochemicals ((1.38±0.08)–(84.2±0.2) %). In the present study, the results revealed that quercetin, curcumin, apigenin and luteolin could be promising QS and biofilm inhibitory agents against the *C. violaceum* 12472 biosensor system. Our findings also suggest that all the phytochemicals, especially curcumin, quercetin and pyrogallol, might be anti-pathogenic agents against *P. aeruginosa* PAO1 infections due to the ability to control QS. However, more comprehensive studies at the molecular level, explaining their anti-QS mechanisms, need to be conducted to confirm these results and identify the genes involved.

Key words: phytochemicals, anti-quorum sensing, *Chromobacterium violaceum* ATCC 12472, *Pseudomonas aeruginosa* PAO1, antibiofilm activity

INTRODUCTION

Chromobacterium violaceum obtained from water, soil, and human skin is a facultative anaerobic, Gram-negative opportunist, which often produces violacein, a characteristic purple, water-insoluble pigment with antibacterial activity. It causes severe morbidity and mortality associated with infections such as bacteremia and abscesses. It is also resistant to multiple antimicrobials, thereby causing recurrent infections (*1–3*). The production of the violacein pigment in *C. violaceum* is controlled by quorum sensing (QS), a process of bacterial cell-cell communication in which cells regulate the transcription of the specific genes responsible for the production of antibiotics, biofilm differentiation, cell division, bioluminescence and other processes (*4,5*).

The QS systems of Gram-negative bacteria are composed of LuxI-type autoinducer synthases that synthesize specific acylated homoserine lactone (AHL) autoinducers and the AHL binds specifically to LuxR receptor protein to trigger specific gene expressions (*6*). For example, *C. violaceum* ATCC 12472 produces and responds to cognate autoinducer molecules

C6-AHL and C4-AHL to induce the production of the violacein pigment (*7*). The compounds preventing the violacein production by this pathogen, without any bacterial inhibition, could be promising QS inhibitors, capable of attenuating bacterial pathogenicity with a lower risk of resistance development than in the case of antibiotics (*8*). On the other hand, *Pseudomonas aeruginosa* is the most prevalent and important opportunistic human pathogen, causing for example fatal lung disease in patients with cystic fibrosis. It can display virulence partially owing to its motility, which plays a significant role in its colonization in various environments, the attachment of the bacteria to surfaces, and biofilm formation. Moreover, the bacterium uses different types of QS signal molecules to synchronize particular gene expressions, including those involved in its biofilm formation and virulence (*9–11*).

The overuse of conventional antibiotics has resulted in the emergence of diseases caused by multi-resistant bacteria. The disadvantages of conventional antimicrobials include their natural selective pressure and failure to treat infections caused by bacterial biofilms (*12*). Compared to conventional antimicrobials, plant-derived compounds, especially bioactive phytochemicals, are not generally associated with many side effects, and they have a significant anti-infective potential against infectious diseases (*13*). Therefore, many bioactive compounds in the form of plant phenols have been used extensively as antimicrobials for decades in traditional medicine (*14*). In addition, the inhibition of bacterial QS system is being evaluated as a new target for developing anti-infective therapies since blocking of QS would weaken virulence of the pathogens, making them more susceptible to treatment and facilitating easy clearance by host defence mechanisms (*15*). Therefore, research focused on the discovery of novel phytochemicals and plant extracts specifically targeting QS signalling systems and their biofilm inhibition has increased recently (*14–17*). Apart from the development of potent therapeutics, the detection of anti-pathogenic phytochemicals interfering with QS and virulence factor production may reveal promising anti-infective compounds. Hence, the aim of the present study is to comparatively investigate anti-QS and antibiofilm potential of the dietary phytochemicals quercetin, curcumin, apigenin, pyrogallol, gallic acid and luteolin, against *C. violaceum* ATCC 12472 as well as their inhibitory activities against swimming and swarming abilities of *P. aeruginosa* PAO1. A few of the phytochemicals, particularly quercetin and curcumin, have already been reported to act as anti-QS and antibiofilm agents (*6,18–21*); however, this study is the first one to compare their inhibitory effects on QS and biofilm formation by *C. violaceum* 12472, and swimming and swarming motility of *P. aeruginosa* PAO1.

MATERIALS AND METHODS

Bacterial strains and culture conditions

In this study, the wild-type strain *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1 were used as biosensor strains for quorum sensing (QS) and motility assays, respectively. *C. violaceum* 12472 was a kind gift from Prof. Dr Robert J.C. McLean (University of Texas, TX, USA), and *P. aeruginosa* PAO1 was also a kind gift from Daniel Lopez, PhD (National Centre for Biotechnology (CNB), Autonomous University of Madrid, Madrid, Spain). The strains were routinely grown in Luria-Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract and 1 % NaCl (Sigma-Aldrich, Merck, St. Louis, MO, USA) medium; pH=7.0). Flasks with *C. violaceum* and *P. aeruginosa* PAO1 were incubated at 30 and 37 °C for 24 h, respectively.

Preparation of dietary phytochemicals

The dietary phytochemicals (quercetin, curcumin, apigenin, pyrogallol and luteolin) were purchased from Sigma-Aldrich, Merck. Gallic acid and all solvents were from Merck (Darmstadt, Germany). The stock solutions of apigenin and luteolin were prepared in 10 % dymethylsulphoxide (DMSO), those of curcumin and quercetin in 50 % methanol, and pyrogallol and gallic acid were dissolved in distilled water. The final volume fraction of DMSO or methanol was adjusted to ≤0.5 %, which did not have any discernible effect on the growth of *C. violaceum* 12472. The stock solutions were prepared daily and diluted to the desired volume fraction immediately prior to use. The control groups were 0.5 % DMSO, 0.5 % methanol and the bacteria cultured in LB broth.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) values of the dietary phytochemicals were determined by the broth microdilution test in 96-well plates as previously described (*22*) with some modifications. A volume of 100 µL of the stock solutions of the phytochemicals (50 % *V/V*) was added to an equal volume of LB broth, and twofold serial dilutions (1500, 750, 375, 187.5, 93.75 and 46.87 µg/mL) were prepared in the microtiter plate. Overnight cultures of *C. violaceum* 12472 and *P. aeruginosa* PAO1 were adjusted to the absorbance value $A_{600 \text{ nm}}$=0.4 using sterile broth, and 100 µL of each culture were added to the plates, after which the plates were incubated at 30 and 37 °C for 24 h, respectively. Inhibition of the bacterial growth in the wells containing the phytochemicals was assessed by a comparison with the growth in blank control wells. Every experiment included negative (medium, 0.5 % DMSO, and 0.5 % methanol) and positive control (medium including the inoculum). The MIC was recorded as the lowest concentration at which there was no visible growth of the bacteria. The MIC assay was repeated at least twice. The subliminal inhibitory concentrations (sub-MICs) of each phytochemical (46.87–750 µg/mL) were tested for the assessment of anti-QS and antibiofilm activity.

Quorum sensing inhibition

Qualitative anti-QS activity: Disc diffusion method

The standard disc diffusion assay, with few modifications, was used for the detection of the anti-QS potential of the

dietary phytochemicals using the wild-type pigmented biosensor strain *C. violaceum* 12472 (*23*). It was grown in LB broth or on the LB agar (1.2 % *m/V*). A volume of 5 mL of molten LB agar (0.3 % *m/V*) was inoculated with 50 µL of the *C. violaceum* 12472 culture grown overnight in LB broth. The agar solution with the culture was immediately poured over the surface of LB agar plates. A volume of 20 µL of each phytochemical solution (46.87, 93.75, 187.5, 375 and 750 µg/mL) was pipetted on sterile paper disks (6 mm diameter; Bioanalyse®, Ankara, Turkey), which was placed on the solidified agar. The plates were incubated overnight at 30 °C and examined for violacein pigment production. QS inhibition, in sub-MIC values, was detected by a ring of colourless but viable cells around the disks. The measurements were made from the outer edge of the disks to the edge of the zones suggesting anti-QS inhibition. Controls were 0.5 % DMSO and 0.5 % methanol. This experiment was carried out at least three times.

Quantitative anti-QS activity: Violacein inhibition

Inhibitory potential of the dietary phytochemicals on the violacein pigment production was also measured spectrophotometrically using the method of Blosser and Gray (*24*), with a few modifications. Quantitative evaluation of anti-QS activity of the phytochemicals was carried out based on their ability to inhibit the production of the purple pigment violacein by *C. violaceum* 12472. Briefly, the phytochemicals were added to 200 µL of bacterial culture (in LB broth) at the sub-MIC concentrations (46.87, 93.75, 187.5, 375 and 750 µg/mL) and incubated at 30 °C until complete pigmentation was achieved in the blank, *i.e.* untreated culture. First, 200 µL of treated and untreated cultures were placed in an Eppendorf tube and lysed by addition of 200 µL of 10 % SDS, vortexed for 5 s and incubated at room temperature for 5 min. Subsequently, 900 µL of water-saturated butanol (50 mL *n*-butanol mixed with 10 mL distilled water) were added to the cell lysate, followed by vortexing for 5 s and centrifugation at 13 000×*g* for 5 min. The upper (butanol) phase containing the violacein was collected and the absorbance was read at 585 nm in UV-Vis spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). The percentage of violacein inhibition was calculated using the following formula:

$$\text{Inhibition}=(A_{585\,nm}(\text{control})-A_{585\,nm}(\text{test})/A_{585\,nm}(\text{control}))\cdot100 \quad /1/$$

where *A* is the absorbance, controls were 0.5 % DMSO, 0.5 % methanol and the untreated bacteria, and the test was the culture treated with the phytochemicals. The experiments were performed at least in triplicate.

Antibiofilm activity

Biofilm formation in 96-well U-bottom polystyrene microtiter plates (Nunc™, Thermo Fisher Scientific, Waltham, MA, USA) was assayed *via* a previously described, slightly modified method (*25*). An overnight culture of *C. violaceum* 12472 was diluted 1:100 with LB broth and grown for another hour. After the addition of sub-MICs of the phytochemicals (46.87, 93.75, 187.5, 375 and 750 µg/mL), 100 µL of the culture were pipetted into the wells of the microtiter plates and the plates were incubated for 24 h at 30 °C. Then, the medium was removed and washed with 1×PBS buffer three times. The plates were dried at 65 °C in a universal oven (UNB 100; Memmert®, Schwabach, Germany) and then 100 µL of a 1 % *m/V* aqueous solution of crystal violet were added. The stain was allowed to fix at room temperature for 20 min, after which the dye was removed from the wells by washing thoroughly with sterile water. For the quantification of the attached biomass, the bound dye was dissolved with 30 % acetic acid solution, and the absorbance was determined at 595 nm. Inhibitor-mediated reduction of biofilm formation was assessed by comparing it to the positive control without phytochemicals. The biofilm assay was performed three times, and six wells per treatment were used each time.

Swimming and swarming assays

The swimming and swarming motility assays were performed using a previously described, slightly modified method (*26*). In swimming assay, 5 µL of overnight culture of the *Pseudomonas aeruginosa* PAO1 ($A_{600\,nm}$=0.4) were point inoculated at the centre of an agar medium consisting of 1 % tryptone, 0.5 % NaCl and 0.3 % agar with the sub-MIC concentration of the phytochemicals (93.75 µg/mL). For swarming assays, the agar medium comprised 1 % peptone, 0.5 % NaCl, 0.5 % agar and 0.5 % filter-sterilized D-glucose with the same sub-MIC concentration of phytochemicals (93.75 µg/mL). The plates were then incubated at 37 °C in an upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swimming and swarming zones of the bacterial cells after 16 h compared to the negative controls.

Growth curve assay

To confirm the anti-QS activity of the dietary phytochemicals, the growth curve assay of *C. violaceum* 12472 cultivated in the presence and absence of phytochemicals was performed. Overnight culture of the bacteria (1 %; $A_{600\,nm}$=0.4) was inoculated in a 250-mL Erlenmeyer flask containing 50 mL of LB broth supplemented with the sub-MIC concentrations (375 and 750 µg/mL) of each phytochemical. The flasks were incubated at 30 °C and 180 rpm in a rotary shaker (KS300; IKA®-Werke GmbH & Co. KG, Staufen, Germany). The cell density was measured by UV-Vis spectrophotometer (UV-1800; Shimadzu) at 1-hour intervals up to 20 h. The control was the bacteria without the treatment with phytochemicals (*19*).

Statistical analysis

The results were analysed using SPSS (Statistical Package for the Social Sciences) v. 20.0 (*27*) and expressed as mean value±standard deviation (S.D.). The differences between the

control and test samples were analysed using *t*-test and one--way ANOVA. Differences at p<0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Minimum inhibitory concentration of dietary phytochemicals against test strains

MIC was determined for each of the dietary phytochemicals at the concentrations ranging from 1500 to 46.87 µg/mL against *Chromobacterium violaceum* 12472. The MIC value of gallic acid was found to be ≥1500 µg/mL. This result corroborates well the findings of Borges *et al.* (*12*)*,* who reported the MIC value of gallic acid of >1000 µg/mL for the same strain. The effect of phenolic acids on the physicochemical properties of bacterial cell surface has shown that these compounds, in particular gallic acid, change bacterial hydrophobicity. As known, phenolic acids alter the polar, nonpolar and electron acceptor components of bacterial cells (*28*).

In addition to these results, the MIC values of curcumin and pyrogallol were found to be 1500 µg/mL and the MIC values of quercetin, luteolin and apigenin were found to be 750 µg/mL. Gopu *et al.* (*6*) found that the MIC value of quercetin was 120 µg/mL against *C. violaceum* CV026. This result differs from our data, which may be due to the use of different methods employed to detect the MIC values, variations in the preparation of the solutions of phytochemicals and/or due to the use of different biosensor strains. In our study, we believe that dietary phytochemicals affect bacterial biochemical activities responsible for bacterial growth.

Phytochemicals are known to have strong antimicrobial effects, which mainly cause structural or functional damage to the bacterial cell membrane (*29*). They could also show different target mechanisms of antimicrobial activity on bacterial cells, such as the degradation of the cell wall, the leakage of the cell contents, the depletion of the proton motive force, or cytoplasmic protein coagulation or inhibition (*30*). Ohemeng *et al.* (*31*) and Mirzoeva *et al.* (*32*) reported that quercetin inhibited DNA gyrase and disrupted the bacterial membrane potential. Sorrentino *et al.* (*33*) also reported that gallic acid caused irreversible changes in permeability profile, rupture and pore formation of the bacterial cell membranes. In our study, the dietary phytochemicals may use one of the target mechanisms to inhibit the growth of *C. violaceum* 12472.

Determination of anti-QS activity by disc diffusion method

The production of the purple pigment violacein in *C. violaceum* is controlled by QS (*4*). Loss of the violacein is a hallmark of QS inhibition in *C. violaceum* 12472 by the dietary phytochemicals. The phytochemicals showed promising anti-QS activity, and a white opaque zone of inhibition was observed in the biosensor plate containing the reference strain *C. violaceum* 12472 (**Fig. 1**). The sub-MICs of quercetin, luteolin and apigenin were found to be <750 µg/mL, and of curcumin, pyrogallol and gallic acid <1500 µg/mL. The sub-MICs of each tested phytochemical ranged from 46.87 to 750 µg/mL. As shown in **Table 1**, the anti-QS activity was concentration-dependent, showing an increase in the diameter of the QS inhibition zones with increasing concentrations of the phytochemicals (p<0.05). Among all the phytochemicals screened for the QS inhibition, curcumin, quercetin, luteolin and apigenin exhibited the anti-QS activity, but pyrogallol and gallic acid did not. Al-Hussaini and Mahasneh (*34*) reported the QS inhibition zones for different herbal extracts with diameters 8–10.5, 13 and 18 mm, corresponding
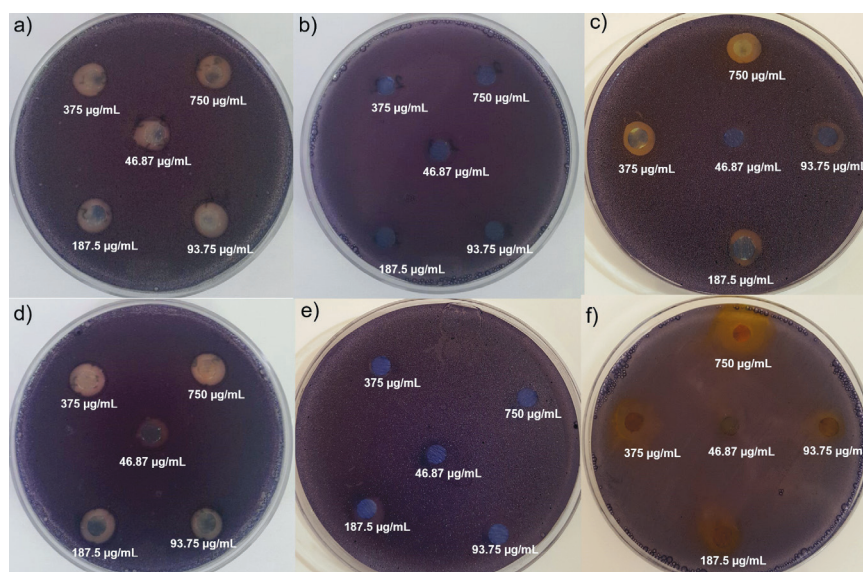

Fig. 1. The anti-quorum sensing activity of the dietary phytochemicals at concentrations from 46.87 to 750 µg/mL against biosensor strain *Chromobacterium violaceum* ATCC 12472 *via* disk diffusion method*:* a) luteolin, b) gallic acid, c) quercetin, d) apigenin, e) pyrogallol, and f) curcumin

Table 1. Zone of violacein inhibition obtained with the subminimal inhibitory concentrations (46.87–750 µg/mL) of the dietary phytochemicals

γ(phytochemical)/(µg/mL)	d(inhibition zone)/mm					
	CR	QT	PG	GA	LT	AP
46.87	(7.0±0.3)[a]	(7.5±0.1)[)a]	NA	NA	(9.1±0.1)[a]	(9.3±0.7)[a]
93.75	(8.5±0.0)[b]	(8.0±0.1)[b]	NA	NA	(10.1±0.4)[b]	(10.3±0.7)[b]
187.5	(9.7±0.1)[c]	(9.5±0.7)[c]	NA	NA	(12.3±0.6)[c]	(11.5±0.5)[c]
375	(12.5±0.3)[d]	(12.0±0.2)[d]	NA	NA	(13.6±0.5)[d]	(13.0±0.1)[d]
750	(13.3±0.1)[e]	(13.3±0.5)[)e]	NA	NA	(14.2±0.1)[e]	(14.3±0.0)[e]

CR=curcumin, QT=quercetin, PG=pyrogallol, GA=gallic acid, LT=luteolin, AP=apigenin; NA=no activity. The values in the same column with different letters in superscript are significantly different (p<0.05)

to weak, moderate and strong anti-QS activity, respectively. In our study, the QS inhibition zones (**Table 1**) were found for curcumin ((7.0±0.3)–(13.3±0.1) mm), quercetin ((7.5±0.1)–(13.3±0.5) mm), luteolin ((9.1±0.1)–(14.2±0.1) mm) and apigenin ((9.3±0.7)–(14.3±0.0) mm) at the concentration range of 46.87–750 µg/mL. At low concentrations (46.87 and 93.75 µg/mL), these phytochemicals had weak anti-QS activities. In addition, curcumin ((9.7±0.1) mm) and quercetin ((9.5±0.7) mm) exhibited weaker anti-QS activities than luteolin ((12.3±0.6) mm) and apigenin ((11.5±0.5) mm) at 187.5 µg/mL. However, curcumin ((12.5±0.3) mm), luteolin ((13.6±0.5) mm) and apigenin ((13.0±0.1) mm) showed stronger anti-QS activities than quercetin ((12.0±0.2) mm) at 375 µg/mL (p<0.05). At the highest sub-MIC (750 µg/mL), curcumin demonstrated the highest QS inhibition (the diameter of zone of inhibition being (13.3±0.1) mm; **Table 1**). The concentration-dependent anti-QS activity of the phytochemicals observed in this study is in agreement with some of the findings reported by Borges *et al.* (*12*), Husain *et al.* (*15*), and Vasavi *et al.* (*20*).

Determination of quantitative anti-QS activity by violacein inhibition

To confirm the QS inhibitory activity of the dietary phytochemicals, the extraction and the quantification of the violacein from *C. violaceum* 12472 culture was also performed in the presence and absence of the phytochemicals (curcumin, quercetin, pyrogallol, luteolin, gallic acid and apigenin) at the same sub-MIC levels (46.87–750 µg/mL). The results showed a concentration-dependent inhibition of the violacein production by curcumin, quercetin, luteolin and apigenin. Compared to the control, all concentration ranges of curcumin, quercetin, luteolin and apigenin (46.87–750 µg/mL) demonstrated a significant drop in the violacein content of *C. violaceum* 12472, without the inhibition of bacterial growth (p<0.05). As shown in **Fig. 2**, curcumin, quercetin, luteolin and apigenin inhibited the violacein production ((14.0±0.3)–(88.2±0.1) %, (11.1±0.1)–(55.03±0.07) %, (37.49±0.08)–(59.48±0.08) % and (36.3±0.2)–(58.95±0.08) %) in a concentration-dependent manner. Among all the phytochemicals, curcumin exhibited the most powerful QS inhibitory effect ((54.7±0.3), (72.2±0.2) and (88.2±0.1) %) at 187.5, 375 and 750 µg/mL. At all concentrations, luteolin and apigenin demonstrated stronger violacein inhibition than quercetin (**Fig. 2**). The results revealed

that the percentage of inhibition of QS by apigenin and luteolin was similar at the tested concentrations. This may be because apigenin and luteolin are the flavonoid compounds with similar structures. Thus, the QS inhibitory effect of the phytochemicals was found to be in the following order: curcumin>apigenin>luteolin>quercetin.
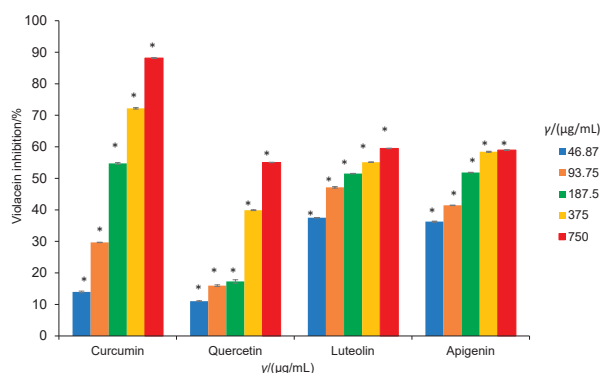

Fig. 2. Quantitative analysis of violacein inhibition in *Chromobacterium violaceum* ATCC 12472 by the dietary phytochemicals at the subminimal inhibitory concentrations (46.87–750 µg/mL). Data are presented as a percentage of violacein inhibition. Mean values of triplicate independent experiments and S.D. are shown. The control groups were: 0.5 % DMSO, 0.5 % methanol and the bacteria cultured in Luria-Bertani broth. *Statistically different from the control (p<0.05)

Brackman *et al.* (*35*) found that curcumin inhibited violacein production by (18±12) % in *C. violaceum* CV026 at a concentration of 184 µg/mL. In our findings, curcumin inhibited violacein production by (54.7±0.3) % at 187.5 µg/mL. In addition, in the present study, the violacein inhibition by 750 µg/mL curcumin ((88.2±0.1) %) is comparable with that of Packiavathy *et al.* (*19*), who reported 89 % violacein inhibition in *C. violaceum* CV026 when treated with 100 µg/mL curcumin. Moreover, the violacein inhibition by quercetin at the maximum sub-MIC of 375 µg/mL ((39.9±0.1) %) is comparable with that of Gopu *et al.* (*6*), who reported that quercetin exhibited maximum violacein inhibition of 83.23 % in *C. violaceum* CV026 at a concentration of 80 µg/mL. Similarly, 50 % inhibition of the violacein production in *C. violaceum* 12472 by quercetin (50 µg/mL) was reported by Vasavi *et al.* (*20*) using a different method.

Apigenin and luteolin inhibited violacein ((58.4±0.2) and (55.13±0.04) %) at 375 µg/mL. Vandeputte *et al.* (*36*) reported that apigenin and luteolin at the concentration of 4 mM (approx. 1.2 mg/mL) exhibit no QS inhibiton, but a bactericidal or bacteriostatic activity on *C. violaceum* CV026. Our study showed that apigenin and luteolin at the sub-MICs of 375, 187.5, 93.75 and 46.87 µg/mL showed anti-QS activity on *C. violaceum* 12472 without inhibition of bacterial growth (**Fig. S1**, **Fig. 1a**, **Fig. 1d** and **Fig. 2**).

Pyrogallol and gallic acid did not exhibit the anti-QS activity when evaluated by the disk diffusion method. Therefore, the percentage of the violacein inhibition by these phytochemicals was evaluated only at the highest concentration (1500 µg/mL), and the obtained results were (76.95±0.02) and (58.76±0.05) % for pyrogallol and gallic acid respectively (data not shown). Considering that no QS inhibition zones were detected on the agar, these results were observed solely due to the inhibition of bacterial growth since gallic acid and pyrogallol only inhibited microbial growth and not the violacein synthesis. The obtained results are also in agreement with those of Borges *et al.* (*12),* who reported that gallic acid exhibited no anti-QS activity on *C. violaceum* biosensor systems.

Previous research of Borges *et al.* (*12,37*) also revealed that as a sustainable source of new broad-spectrum antimicrobial products, gallic acid can cause irreversible changes in some Gram-negative and Gram-positive bacteria. In addition, Tinh *et al.* (*38*) reported that pyrogallol also exhibited the antibacterial activity against *Vibrio parahaemolyticus*. Although pyrogallol is known as the phytochemical with anti-QS activity and the ability to inhibit autoinducer-2 (AI-2)-mediated QS in *Vibrio harveyi* (*39,40*), it has been revealed in further investigations that this AI-2-mediated QS inhibition is a side effect of the peroxide-producing activity of this compound rather than true QS inhibition (*41*). Our findings also show that pyrogallol does not inhibit QS system of the *C. violaceum* 12472 biosensor strain.

Determination of antibiofilm activity of dietary phytochemicals

In the present study, the biofilm inhibition potential of all the phytochemicals (curcumin, quercetin, pyrogallol, luteolin, gallic acid and apigenin) was evaluated at the same sub--MIC range (46.87–750 µg/mL) (**Fig. 3**). All the phytochemicals exhibited a significant concentration-dependent antibiofilm activity (p<0.05). Gallic acid showed the weakest biofilm inhibition (from (1.38±0.08) to (9.57±0.06) %) at 46.87–750 µg/mL, although it did not possess antimicrobial activity against *C. violaceum* 12472 at <1500 µg/mL. Compared to the other compounds (pyrogallol, apigenin, quercetin and luteolin), curcumin showed the weakest biofilm inhibition ((11.0±0.5) and (26.7±0.3) % at 46.87 and 93.75 µg/mL, respectively). Our results are comparable with the results of Packiavathy *et al.* (*18),* who found that curcumin (>100 µg/mL) inhibited the biofilm formation of *Vibrio harveyi* (69 %), *Vibrio parahaemolyticus* (56 %) and *Vibrio vulnificus* (79 %), without affecting their

planktonic growth. Packiavathy *et al.* (*19*) also reported that curcumin (100 µg/mL) efficiently inhibited the biofilm biomass growth of some uropathogens, including *Escherichia coli* (52 %), *Pseudomonas aeruginosa* PAO1 (89 %), *Proteus mirabilis* (52 %) and *Serratia marcescens* (76 %). In addition, the combined effects of curcumin and honey, as a traditional medicine, and epigallocatechin gallate, as a green tea polyphenol, were reported to enhance significantly the inhibition of biofilm formation in wastewater bacteria (52 to 99 %) and *P. aeruginosa* PAO1 (20 to 94.6 %) (*21,42*). Therefore, the antibiofilm activity of curcumin may vary depending on the type of strain, the method used and/or its combination with some other constituent. Our results (**Fig. 3**) showed that although curcumin exhibited a weak biofilm inhibition potential at concentrations of 46.87 and 93.75 µg/mL, at higher sub-MICs of 187.5, 375 and 750 µg/mL, it had a strong concentration-dependent activity ((57.7±0.4), (75.6±1.0) and (84.2±0.2) %; p<0.05). **Fig. 3** shows that the biofilm inhibitory potentials of the phytochemicals at concentrations of 46.87 and 93.75 µg/mL increased in the following order: gallic acid<curcumin< pyrogallol<quercetin<apigenin<luteolin. At these concentrations, luteolin had the maximum biofilm inhibition values of (46.94±0.05) and (60.0±0.2) %, respectively. Quercetin, apigenin and pyrogallol also significantly reduced (p<0.05) the biofilm formation of *C. violaceum* 12472 (**Fig. 3**).
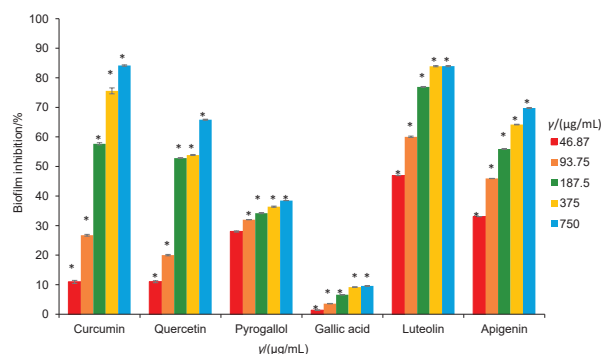

Fig. 3. The percentage of biofilm inhibition in *Chromobacterium violaceum* ATCC 12472 by the dietary phytochemicals at the subminimal inhibitory concentrations (46.87–750 µg/mL). Data are presented as a percentage of violacein inhibition. Mean values of triplicate independent experiments and S.D. are shown. The control groups were: 0.5 % DMSO, 0.5 % methanol and the bacteria cultured in Luria-Bertani broth. *Statistically different from the control (p<0.05)

Pyrogallol showed weak biofilm inhibition with (34.2±0.2), (36.4±0.2) and (38.5±0.0) % at higher sub-MICs of 187.5, 375 and 750 µg/mL, respectively (**Fig. 3**). Thus, the biofilm inhibitory potentials of all the phytochemicals at higher sub-MICs of 187.5 and 375 µg/mL increased in the following order: gallic acid<pyrogallol<quercetin<apigenin<curcumin<luteolin. Luteolin had the maximum biofilm inhibition ((46.94±0.05)–(83.9±0.2) %) at the sub-MIC values of 46.87 to 375 µg/mL. Our results are in accordance with the findings of Vikram *et al.* (*43),* who reported that quercetin and apigenin reduced the biofilm formation in *V. harveyi* and *E.*

coli O157:H7. In addition, Shehzad *et al.* (*44*) and Lee *et al.* (*45*) also reported that curcumin, pyrogallol and apigenin were effective polyphenols against the biofilm formation of *Candida albicans*.

Swimming and swarming motility

In this study, the motility (swimming and swarming behaviour) of *P. aeruginosa* PAO1 in the presence and absence of the dietary phytochemicals at the sub-MIC of 93.75 µg/mL was evaluated using agar plates. Our results demonstrate that all the phytochemicals significantly (p<0.05) blocked the swimming and swarming motility of *P. aeruginosa* PAO1 without impairing its growth capacity. Its swimming and swarming motilities after the treatment with the phytochemicals were observed to be significantly poor (p<0.05; **Table 2**). Compared to the control, curcumin exhibited the maximum reduction in the swimming and swarming motility assays. These results are in accordance with the previous reports of Packiavathy *et al.* (*19*) and Jadaun *et al.* (*42*), who observed a remarkable decrease in the swimming and swarming motility of *P. aeruginosa* PAO1 when treated with curcumin. **Table 2** also indicates the inhibition of swimming and swarming behaviour of *P. aeruginosa* PAO1 by quercetin.

Table 2. Effect of the dietary phytochemicals at the subminimal inhibitory concentration (93.75 µg/mL) on swimming and swarming motility of *Pseudomonas aeruginosa* PAO1

Phytochemical	*l*(migration)/mm	
	Swimming motility	Swarming motility
Control	(44.0±3.5)[a]	(32.0±1.7)[a]
CR	(11.3±0.4)[b*]	(12.5±0.7)[b*]
QT	(15.7±0.6)[c*]	(14.5±0.7)[c*]
PG	(19.3±1.2)[d*]	(16.0±1.4)[d*]
GA	(33.3±0.6)[e*]	(25.5±2.1)[e*]
LT	(28.6±0.4)[f*]	(23.5±1.3)[f*]
AP	(26.4±0.5)[g*]	(17.5±0.4)[g*]

CR=curcumin, QT=quercetin, PG=pyrogallol, GA=gallic acid, LT=luteolin, AP=apigenin. The data represent the mean values of three independent experiments. The values in the same column with different letters in superscript are significantly different (p<0.05). *Statistically different from the control (p<0.05)

Our findings are consistent with the reports of Vasavi *et al.* (*20*), who found that the flavonoid fraction of *Psidium guajava* leaves included an active compound, quercetin-3-O-arabinoside, inhibits the swarming motility of *P. aeruginosa* PAO1. In addition, Gopu *et al.* (*6*) reported that quercetin significantly reduced the swimming and swarming behaviour of foodborne isolates of *Pseudomonas aeruginosa* PUFSTb04 at the concentration of 80 µg/mL. **Table 2** shows that the inhibition potential of the swimming and swarming motilities of the bacterial strain by the investigated phytochemicals decreased in the following order: curcumin>quercetin>pyrogallol>apigenin>luteolin>gallic acid.

The obtained results clearly indicate that curcumin and quercetin exhibited the most powerful inhibition of the motility. The results also suggest a correlation between the inhibition of the swimming and swarming motility for each phytochemical.

Bacterial growth curve

The bacterial growth curve revealed that the sub-MIC concentrations of dietary phytochemicals used in this study (375 and 750 µg/mL) did not have a growth inhibitory effect on *C. violaceum* 12472 (**Fig. S1**).

CONCLUSIONS

This study investigated the inhibitory potentials of six dietary phytochemicals (curcumin, quercetin, apigenin, luteolin, gallic acid and pyrogallol) against the violacein pigment production that is controlled by quorum sensing (QS) and biofilm formation in *Chromobacterium violaceum* ATCC 12472 biosensor system, and their impact on swimming and swarming behaviour of *Pseudomonas aeruginosa* PAO1. Our results also demonstrated that all the phytochemicals, except pyrogallol and gallic acid, inhibited the violacein production. Moreover, the biofilm formation was significantly hindered by all the phytochemicals at each of the sub-MICs in the range of 46.87–750 µg/mL (p<0.05). Although some of the phytochemicals, especially curcumin and quercetin, possess QS and biofilm inhibitory potentials, this study reveals that apigenin and luteolin could also inhibit QS and biofilm formation. Our results also show that all the phytochemicals, especially curcumin, quercetin and pyrogallol, may be used as anti-pathogenic agents, particularly against *P. aeruginosa* PAO1, owing to QS control; however, more detailed experiments should be performed to find out their anti-QS mechanisms. Since these phytochemicals are the active compounds in foods such as onion, broccoli, apples, avocado, mango, banana, parsley, cabbage, carrots, mustard, pepper and radish, the consumption of these foods might be beneficial in the treatment of bacterial infections. In addition, the studies of QS inhibitory potentials of the phytochemicals, especially apigenin and luteolin, against other biosensor strains, and the antibiofilm effect of the phytochemicals against different pathogens are scarce in the literature. Therefore, we suggest that more detailed experiments be conducted to reveal the anti-QS mechanisms and the pharmaceutical potential of the phytochemicals used in this study to confirm these results at the molecular level.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

All supplementary material is available at www.ftb.com.hr.

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