

Diarylheptanoids from *Alnus viridis* ssp. *viridis* and *Alnus glutinosa*: Modulation of Quorum Sensing Activity in *Pseudomonas aeruginosa*

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Key words

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Abstract

Diarylheptanoids from the barks of *Alnus viridis* ssp. *viridis* (green alder) and *Alnus glutinosa* (black alder) were explored for anti-quorum sensing activity. Chemicals with anti-quorum sensing activity have recently been examined for antimicrobial applications. The anti-quorum sensing activity of the selected diarylheptanoids was determined using two biosensors, namely *Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* CV026. Although all of the investigated compounds negatively influenced the motility of *P. aeruginosa* PAO1, four were able to inhibit biofilm formation of this human opportunistic pathogen for 40–70%. Three of the diarylheptanoids (**3**, **4**, and **5**) negatively influenced the biosynthesis of pyocyanin, which is under the control of quorum sensing. Platyphyllenone (**7**) and hirsutenone (**5**) were able to inhibit the biosynthesis of violacein in *C. violaceum* CV026, with **5** being able to inhibit the synthesis of both

biopigments. Only one of the tested diarylheptanoids (**1**) was shown to significantly decrease the production of acyl homoserine lactones (AHL) in *P. aeruginosa* PAO1, more specifically, production of the long chain N-(3-oxododecanoyl)-l-HSL. On the other side, four diarylheptanoids (**2–5**) significantly reduced the synthesis of 2-alkyl-4-quinolones, part of the *P. aeruginosa* quinolone-mediated signaling system. To properly assess therapeutic potential of these compounds, their *in vitro* antiproliferative effect on normal human lung fibroblasts was determined, with doses affecting cell proliferation between 10 and 100 µg/mL. This study confirms that the barks of green and black alders are rich source of phytochemicals with a wide range of biological activities that could further be exploited as natural agents against bacterial contaminations and infections.

Supporting information available online at <http://www.thieme-connect.de/products>

Introduction

Linear diarylheptanoids are secondary metabolites consisting of two benzene rings connected with a heptane skeleton (● Fig. 1). The best-known and most investigated member of this group is curcumin [1,2], a compound isolated from *Curcuma longa* L. (Zingiberaceae) almost 170 years ago, whose structure was determined in 1910 [3], but whose medicinal values were known for thousands of years [4]. Beside the genus *Curcuma*, diarylheptanoids are isolated from Zingiberaceae genera *Zingiber* and *Alpinia*, and Betulaceae genera *Betula* and *Alnus*. The first diarylheptanoids from *Alnus* species, yashabushiketol and dihydroyashabushiketol, were isolated from *Alnus firma* Siebold & Zucc. in 1969 and 1970 by Yoshinori Asakawa [5,6]. Among the

best-known linear diarylheptanoids from *Alnus* species are oregonin, platyphyllaside, hirsutenone, and platyphyllenone (● Fig. 1). They are isolated from black and gray alder (oregonin, platyphyllaside, hirsutenone) and green alder (platyphyllenone) and are well known for their anti-cancer, anti-inflammatory, and antioxidative properties [7–12].

Green alder [*Alnus viridis* (Chaix) DC.] is a bush, 3–5 m tall, found in the mountains of central Europe (Alps) and the Balkan peninsula [13]. In Serbia, *Alnus viridis* (Chaix) DC. ssp. *viridis* can be found only in the east regions (Mt. Stara Planina), mostly near creeks, at an altitude of 1300–2100 m [14]. Black alder [*Alnus glutinosa* (L.) Gaertn.] is a tree, up to 25 m tall, that thrives in moist soil, near creeks and rivers, at an altitude up to 600 m, and is widespread in Europe, the Mediterranean,

southeastern Asia, and the Caucasus Mountains. In Serbia, it can be found mostly in the central and west regions, always near creeks and rivers [14].

Green and black alders have been the focus of our research as exceptionally rich sources of phytochemicals [15,16]. Investigated diarylheptanoids were previously isolated from the CHCl₃/MeOH (1 : 1) extracts of the barks of the green and black alders and were chemically characterized [10,17,18]. They were the most common diarylheptanoids in these extracts. Poor to moderate antibacterial activity previously observed with these compounds [19] led us to examine their activity on bacterial communication within this study. Bacterial cell-to-cell signaling, so-called quorum sensing (QS), is involved in the regulation of virulence expression and biofilm development, traits that are crucial for pathogenicity and the interaction with colonization of eukaryotic hosts [20,21]. Among gram-negative bacteria, QS is frequently mediated by N-acyl-L-homoserine lactone (AHL) signal molecules [22–24]. Virulence factors of important human pathogens such as *P. aeruginosa* are regulated in this manner [25,26]. *P. aeruginosa* has two AHL-mediated QS systems, namely *las* and *rhl*, and one quinolone (AHQs)-mediated signaling system [25,26]. The *las* system consists of a LasR regulator and the cognate autoinducer N-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL), while the *rhl* system consists of RhlR and the autoinducer N-butyryl-L-HSL (C4-HSL). These two AHL-mediated systems regulate the expression of many virulence factors, including LasA protease, phospholipase, exotoxin A, pyocyanin, rhamnolipids, and elastase, and also regulate the development of biofilms, while AHQs signaling, apart from regulating numerous virulence genes in common with AHLs, regulates genes involved in iron scavenging [25,26]. Compounds that attenuate virulence, without killing or a pronounced effect on growth, offer an alternative therapeutic target with less possibility of resistance development [24]. Inhibitors can disrupt QS in various ways, such as by acting as enzymes (e.g., AHL-lactonase or AHL-acylase) that destroy signal molecules, as enzymes that degrade LuxR protein, the receptor for QS signaling molecules, or as AHL mimics that compete and block signal molecules [27,28].

Plants are constantly exposed to bacterial infections. It is therefore logical to expect that plants have developed sophisticated chemical mechanisms to combat pathogens. Indeed, mushrooms [29,30] and various plant extracts have been reported as efficient in bacterial QS inhibiting activity [31–33]. In this study, as a continuation of our studies on diarylheptanoids from plant sources, we have examined the effect of a series of the seven most common diarylheptanoids from black and green alders using two model bioreporter strains: *Pseudomonas aeruginosa* PAO1, including biosensors derived from it, and *Chromobacterium violaceum* CV026. PAO1 is a strain of *P. aeruginosa*, a gram-negative swarming opportunistic pathogen that usually forms persistent biofilms. It is a primary cause in infections associated with cystic fibrosis (CF), burn wounds and the use of catheters and other medical devices, and a major cause of nosocomial infections [34]. Its biofilm forming ability leads to an increased tolerance to antibiotics and the persistence of chronic infections [35,36]. *P. aeruginosa* is often expanded by food, so natural agents suppressing their communication and influencing persistence and virulence are highly sought after. CV026 is a derivative of the gram-negative bacterium *C. violaceum*, commonly found in soil and water [37]. Wild-type *C. violaceum* produces the characteristic purple pigment, violacein, when AHL signaling molecules reach a threshold level [38]. Based on this readily observed pig-

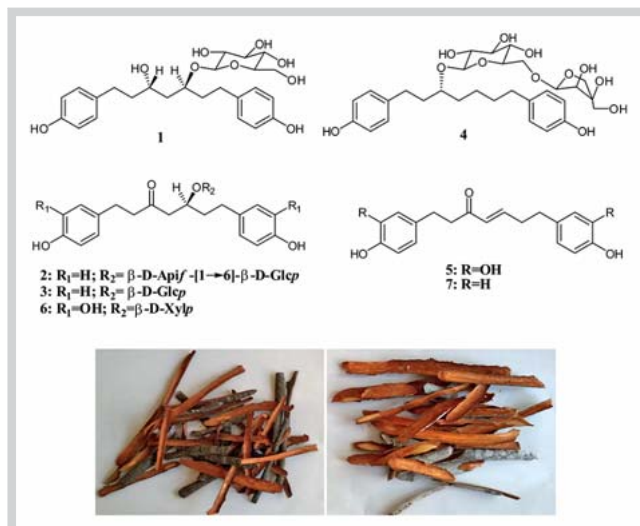


Fig. 1 Investigated diarylheptanoids (top) and barks of black alder (left) and green alder (right). (1S,3R)-3-Hydroxy-5-(4-hydroxyphenyl)-1-[2-(4-hydroxyphenyl)ethyl]pentyl-O-β-D-glucopyranoside (**1**), (5S)-1,7-bis(4-hydroxyphenyl)-5-O-β-D-apiofuranosyl(1 → 6)-β-D-glucopyranosyloxyheptan-3-one (**2**), platyphylloside (**3**), aceroside VIII (**4**), hirsutenone (**5**), oregonin (**6**), and platyphyllenone (**7**). (Color figure available online only.)

mentation phenotype, *C. violaceum* strains can be used to detect various aspects of AHL-mediated QS activity [39]. The CV026 strain harbors a LuxR receptor homologue and a corresponding QS-controlled promoter fused to a violacein production associated gene cluster and it is unable to produce violacein unless exogenous AHLs are supplied [37]. In addition, to further evaluate the safety for potential application of selected diarylheptanoids, *in vitro* antiproliferative activity of these compounds was determined.

Results and Discussion

In this work, diarylheptanoids from the two plant species, green and black alders, were examined for anti-QS activity. Diarylheptanoids from the green alder were (1S,3R)-3-hydroxy-5-(4-hydroxyphenyl)-1-[2-(4-hydroxyphenyl)ethyl]pentyl-O-β-D-glucopyranoside (**1**), (5S)-1,7-bis(4-hydroxyphenyl)-5-O-β-D-apiofuranosyl(1 → 6)-β-D-glucopyranosyloxyheptan-3-one (**2**), platyphylloside (**3**), aceroside VIII (**4**), and platyphyllenone (**7**). Platyphylloside (**3**), hirsutenone (**5**), and oregonin (**6**) were diarylheptanoids originated from the black alder. Platyphylloside (**3**) was isolated from both green and black alder barks (● Fig. 1). The minimal inhibitory concentrations (MICs) of seven investigated diarylheptanoids against *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PAO1 planktonically grown cells were in the range of 125 to 500 μg/mL (Table S1, Supporting Information) and comparable or slightly higher to those previously determined MIC values against *P. aeruginosa* NCTC6750 [17]. Their effect on *P. aeruginosa* PAO1 biofilm formation was tested at subinhibitory concentrations (50% of determined MICs [19] (Table S1, Supporting Information) and results showed that compounds **1–4** had a high potential to inhibit the early formation of biofilms (● Fig. 2). Compound **3** reduced biofilm formation by 40% in comparison to the DMSO control, compound **1** by 60% (similar to the positive control streptomycin), while a reduction in the

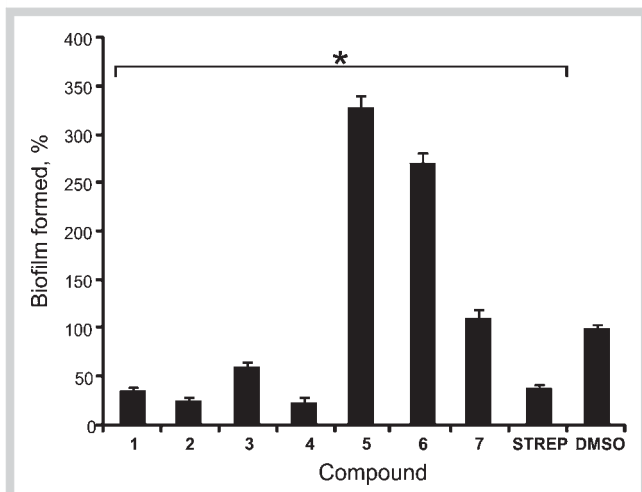


Fig. 2 *P. aeruginosa* PAO1 biofilm formation in the presence of subinhibitory concentrations (0.5 MIC values) of diarylheptanoids and the antibiotic streptomycin (STREP). The biofilm formation in the presence of DMSO was considered to represent 100% biofilm formation. The experiment was done in quintuplicate and repeated two times.

presence of **2** and **4** was around 70%. Compound **7** had no capacity to prevent biofilm formation, and, surprisingly, compounds **6** and **5** exhibited a stimulatory effect with 2.5-fold and 3-fold higher biofilm formation, respectively, at the concentration tested (● Fig. 2).

Apart from QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures: the flagellum and type IV pili [40,41]. The flagellum is responsible for swimming motility, while the type IV pili are responsible for twitching motility [42]. Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* [40,41]. Therefore, we tried to determine if investigated diarylheptanoids influence either one or both motilities (Table S2, Supporting Information). All diarylheptanoids reduced the twitching motility of *P. aeruginosa* (Fig. S1, Supporting Information). Colonies of *P. aeruginosa* PAO1 grown without diarylheptanoids were flat with a rough appearance displaying irregular colony edges, and hazy zones were surrounding these colonies (Fig. S1 J, Supporting Information). Untreated cells grew in a very thin layer. After 2 days of incubation at ambient temperature, colony expansion occurred very rapidly due to twitching motility (Table S2, Supporting Information). Bacteria that were grown in the presence of diarylheptanoids were incapable of producing such twitching zones and had almost round, smooth, regular colony edges, while protrusions were reduced both in size (24–120 μm) and in number (Fig. S1 A–G, Supporting Information), and the colony diameters of swimming zones were also reduced. In comparison, antibiotics such as streptomycin reduced the protrusion (Fig. S1 H, Supporting Information), while the presence of ampicillin, which *P. aeruginosa* PAO1 is resistant to, had no significant effect on protrusion (Fig. S1 I, Supporting Information).

To evaluate the potential of diarylheptanoids as inhibitors of AHL-based QS signal molecules, two biosensor reporter strains were used: *P. aeruginosa* PAO1 and *C. violaceum* CV026. *P. aeruginosa* PAO1 produces a blue-green pigment pyocyanin under the control of AHL, while *C. violaceum* CV026 is a white, violacein negative, double mini-Tn mutant that has no constitutive ability of violacein pigment production, but CV026 can sense exogenously

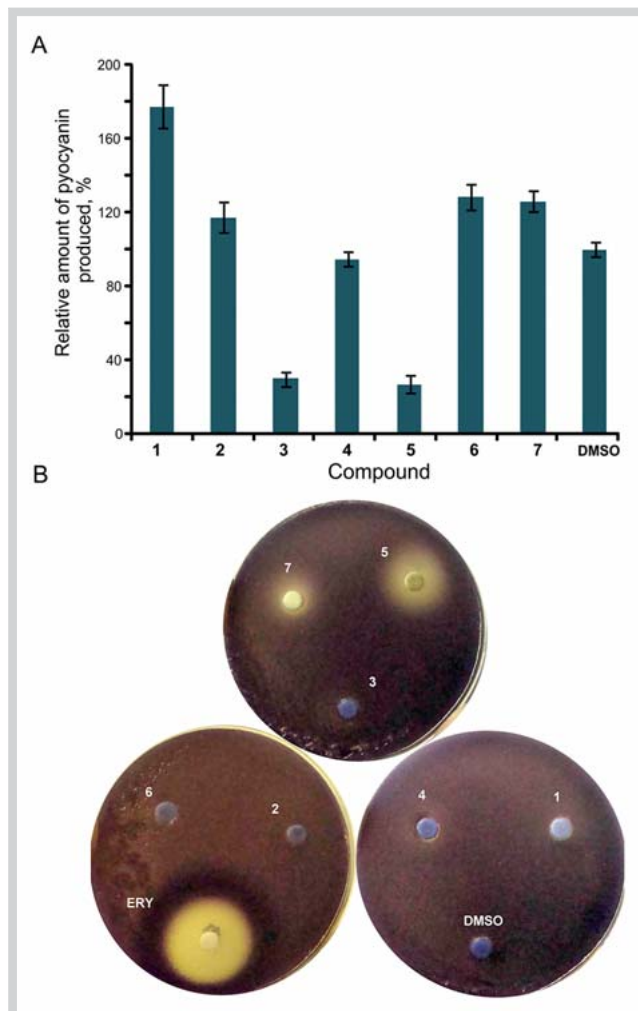


Fig. 3 A Effects of diarylheptanoids (50 μg/mL) on the production of pyocyanin by *P. aeruginosa* PAO1. B Bioassay of inhibition of violacein synthesis in *C. violaceum* CV026 by diarylheptanoids (250 μg/disc). Compounds **5** and **7** showed considerable inhibition of violacein production. A colorless, but not clear zone around the discs indicated anti-QS activity. The negative and positive controls in the bioassay were solvent DMSO and the antibiotic erythromycin (ERY). (Color figure available online only.)

added AHLs and responds by producing purple violacein [37,38,43].

The effect of diarylheptanoids on pyocyanin production in *P. aeruginosa* PAO1 was tested in a flask assay at the concentration of 50 μg/mL (● Fig. 3 A). Of the seven investigated compounds, diarylheptanoids **3** and **5** significantly reduced pyocyanin production by 70% and 73%, respectively, whereas all of the others diarylheptanoids stimulated pyocyanin production (in the range of 17–77%) compared to the control *P. aeruginosa* PAO1 (DMSO). Diarylheptanoids **5** and **7** demonstrated considerable antagonistic activity on QS in CV026 when 100 μg of the test compound was applied per disc. Inhibition of violacein production with **5** and **7** was clearly visible as a colorless halo around the discs (● Fig. 3 B), not as strong as erythromycin, but significant to suggest that these diarylheptanoids could be blocking AHL-QS signaling. Many mechanisms of actions have been proposed to interfere with the QS inhibition, such as inhibition of biosynthesis of autoinducer molecules, inactivation or degradation of the autoinducer, interference with the signal receptor, and inhibition

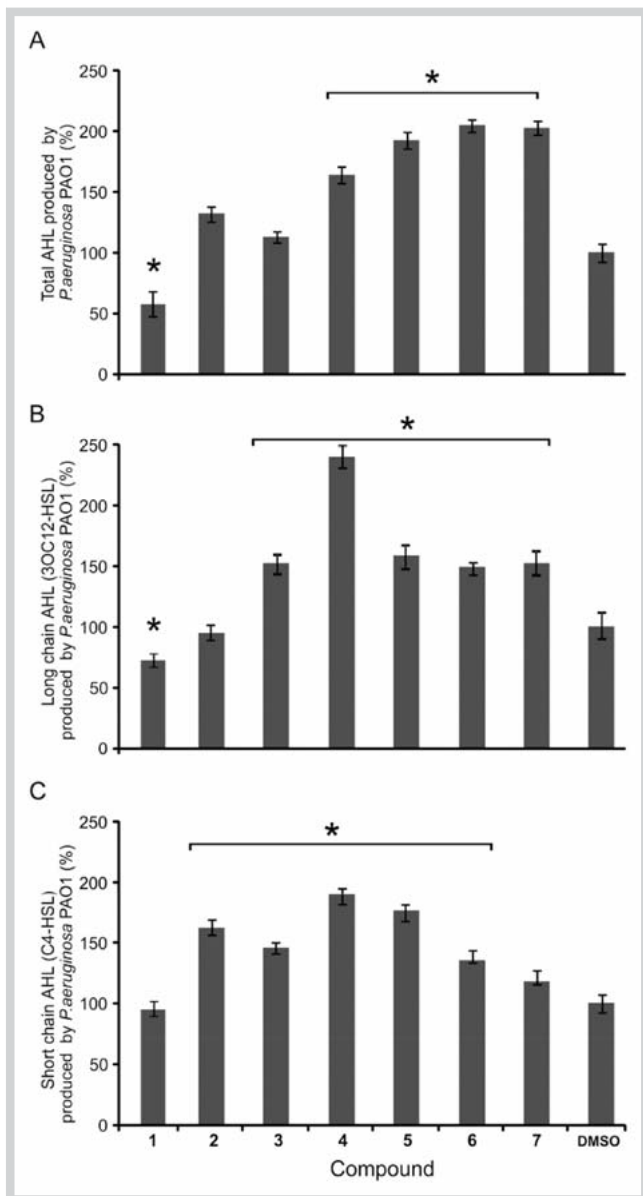


Fig. 4 Effect of diarylheptanoids (50 $\mu\text{g/mL}$) on acyl homoserine lactone (AHL) production in *P. aeruginosa* PAO1. **A** Total AHLs; **B** Long chain (3OC12-HSL) and **C** short-chain (C4-HSL). The PAO1 $\Delta\text{rhlpKD-rhIA}$ reporter strain was used to directly measure the levels of C4-HSL, and the PA14-R3 $\Delta\text{lasIPrsal:luc}$ strain was used to directly measure the levels of 3OC12-HSL in the culture supernatants of *P. aeruginosa* PAO1. *Represents statistically significant differences between compounds. DMSO served as the negative control.

of the genetic regulation system [44]. Hirsutenone (5) had the ability to inhibit synthesis of both biopigments.

To test whether the diarylheptanoids influenced AHL production in *P. aeruginosa* PAO1, we have grown this strain in the presence of 50 $\mu\text{g/mL}$ diarylheptanoid and then performed the extraction of the AHLs. In the assay with *C. violaceum* CVO26 as a reporter organism of QS, five out of seven AHL extracts from *P. aeruginosa* PAO1 manifested an unambiguous modulatory effect on violacein synthesis (Fig. 4). Interestingly, a notable stimulatory effect on overall AHL production in *P. aeruginosa* PAO1, in the range of 150% to 200% compared to the negative control (DMSO; Fig. 4A), was observed when four compounds (4–7) were sup-

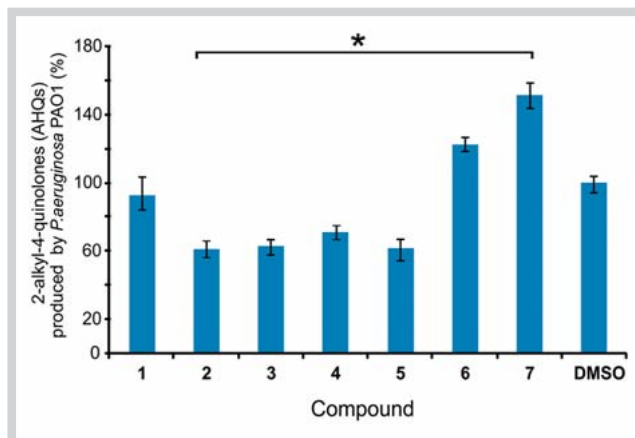


Fig. 5 Effect of diarylheptanoids (50 $\mu\text{g/mL}$) on AHQs production in *P. aeruginosa* PAO1. Detection of AHQs (PQS and HHQ) was performed by the biosensor PAO1 ΔpqqsA mini-CTX luxPpqsA. Extract of *P. aeruginosa* PAO1 grown in the presence of DMSO served as the negative control. *Represents statistically significant differences between compounds. DMSO served as the negative control. (Color figure available online only.)

plied to the medium in these concentrations. When compound 1 was used, it reduced overall AHL production in *P. aeruginosa* PAO1 by 40% (Fig. 4A).

To differentiate the effects of seven diarylheptanoids on *rhl* and *las* QS systems in *P. aeruginosa* PAO1, two biosensors strains were included: PA14-R3 $\Delta\text{lasIPrsal:luc}$ for the detection of a long-chain AHL (3-oxo-C12-HSL) and PAO1 $\Delta\text{rhlpKD-rhIA}$ for the detection of a short-chain AHL (C4-HSL; Fig. 4B–C). Only compound 1 significantly reduced the production of 3-oxo-C12-HSL by 35%, possibly accounting for its biofilm preventing activity (Fig. 2). Most of the other diarylheptanoids stimulated both the production of 3-oxo-C12-HSL and C4-HSL, suggesting that their observed activities on *P. aeruginosa* PAO1 biofilm and biopigment synthesis occurred via different mechanisms of action. *P. aeruginosa*, as some other bacterial species, has multiple interdependent QS systems. It produces diverse 2-alkyl-4-quinolones (AHQs), which act as QS signal molecules and are also involved in the regulation of many virulence factors. PQS has been shown to control the production of multiple virulence determinants, including elastase, pyocyanin, rhamnolipids, and biofilm development [20,25,28,45]. Biosensor strain *P. aeruginosa* PAO1 ΔpqqsA mini-CTX luxPpqsA was used to evaluate the influence of selected diarylheptanoids to AHQs production in *P. aeruginosa* PAO1 (Fig. 5). Compounds 2–5 significantly reduced the production of 2-alkyl-4-quinolones in *P. aeruginosa* PAO1 cultures grown in the presence of these compounds at 50 $\mu\text{g/mL}$ when compared to the negative control (DMSO treated). On the other side, extracts from *P. aeruginosa* PAO1 cultures cultivated with compounds 6 and 7 showed a stimulatory effect of 120% to 150%, respectively (Fig. 5). These results indicated that diarylheptanoids more efficiently modulate the quinolone-mediated QS system in *P. aeruginosa* PAO1. The quinolone system is usually transferred via extremely hydrophobic molecules, usually in membrane vesicles [25,26], so structural differences of selected quite polar diarylheptanoids (Fig. 1) could not account for the observed differences in the QS modulating activity.

In the present study, we have evaluated antiproliferative effects of the seven diarylheptanoids against MRC5 cells line (human lung fibroblasts) using the MTT assay. Doxorubicin was used as a

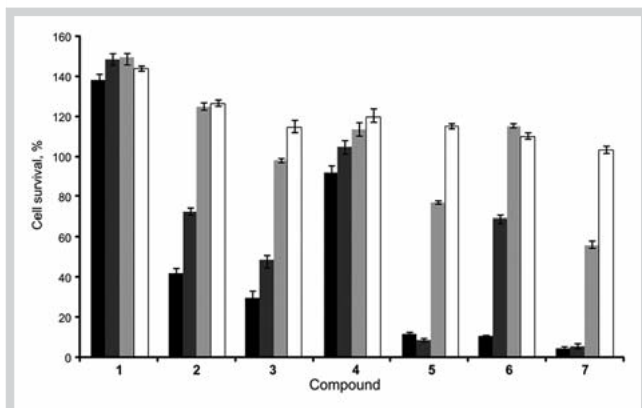


Fig. 6 Antiproliferative activity of diarylheptanoids on the growth of the MRC5 cell line. Cells were treated with 1 □, 10 ■, 50 ■, and 100 ■ µg/mL of each tested compound for 48 h. Doxorubicin was used as a positive control. Viability was measured by the MTT assay. Values are expressed as the percent of cell survival and are based on quadruplicate tests performed twice, with mean values shown and standard deviations depicted by the vertical bars.

positive control. The results of the MTT assay showed that diarylheptanoids could inhibit cell proliferation in a concentration-dependent manner (● Fig. 6). Five tested compounds, 2, 3, and 5–7, were cytotoxic when applied in high concentrations, especially 5–7 exhibited a stronger cytotoxic effect than doxorubicin at the concentrations of 50 µg/mL and 100 µg/mL. They were able to inhibit proliferation of the fibroblasts cell line between 95% and 60%. Also, when supplied in the concentration of 50 µg/mL, compounds 7 and 5 exhibited a cytotoxic effect, inhibiting fibroblast proliferation at about 95%. These five compounds were active towards MRC5 cells with IC_{50} values of 10, 20, 50, 60, and 70 µg/mL, respectively, whereas compounds 1 and 4 did not show any antiproliferative activity at the tested concentrations (● Fig. 6). Therefore, 1 and 4 can be considered safe in concentrations of up to 250 mg/mL (which was the highest concentration included in the screen; results not shown). They also efficiently inhibited formation of *P. aeruginosa* biofilms (● Fig. 2) and successfully inhibited the production of QS signaling molecules (● Fig. 4B and Fig. 5). Thus, these two diarylheptanoids can be further examined for application in antimicrobial therapy.

Platyphylloside (3), previously isolated from the inner bark of *Betula papyrifera* Marshall, exhibited IC_{50} concentrations with three different cell lines A549, DLD-1, and WS1 between 10.3 and 13.8 µM [45]. Similarly, according to literature data, hirsutone (5) was isolated from various plant sources and was found to be cytotoxic against a number of different cell lines including macrophages, HeLa, and the HT-29 human colon cancer cell line [46,47]. This was also in accordance with our previous data that defined 3 and 5 as compounds with anticancer potential, being active against human lung carcinoma cell lines [10].

QS plays a critical role in bacterial virulence and survival, which makes this signaling pathway an attractive target for antimicrobial drug development. This has afforded a novel opportunity to control the activity of gram-negative infecting bacteria by other means than interfering with their growth, posing a lower chance for the development of resistance. We have shown that compounds, particularly diarylheptanoids, interfering with bacterial communication systems are present in the bark of green and black alders. Our data also confirmed that multiple families of

QS pathways interactively regulate different gene expressions in *P. aeruginosa* PAO1, and that although structurally similar, diarylheptanoids cause different effects on phenotypic characteristics under QS control. Overall, selected diarylheptanoids may not have a wide therapeutic window, but further research into their or other diarylheptanoids of plant origin should be carried out in the direction of bacterial anti-QS application.

Material and Methods

Plant material and compounds origin

Diarylheptanoids investigated in this work were previously isolated from the $CHCl_3/MeOH$ (1 : 1) extracts of the black and green alder barks from Serbia [17, 18]. Silica gel column chromatography followed by semipreparative reversed-phase HPLC were the techniques used for isolation. The structures of the diarylheptanoids were elucidated by means of 1D and 2D NMR, IR, UV, and HR-ESI-MS. Prior to investigation of the biological activity, all tested diarylheptanoids were checked for their purity by HPLC (at 280 nm) and 1H NMR, and it was higher than 98%.

Bacterial strains, growth media, and culture conditions

P. aeruginosa ATCC 27853 was obtained from the American Type Culture Collection, while *P. aeruginosa* PAO1, *P. aeruginosa* PAOJP2, *P. aeruginosa* PA14-R3, and PAO1 Δ pqsAmini-CTXlux-PpqsA were kindly provided by Dr. Livia Leoni, Department of Biology, University Roma Tre, Italy. Biosensor strain *C. violaceum* CV026 [37] was provided by Prof. Vittorio Venturi (ICGB, Trieste, Italy). Bacteria were routinely grown in Luria-Bertani (LB) medium [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract] with or without agar (1.5% w/v), then were shaken (180 rpm) at 37°C. Tryptone soy broth (TSB; Oxoid) was used for growth of *P. aeruginosa* PAO1 in the assay of biofilm formation. Mueller-Hinton agar (MH; Oxoid) was used to determine the motility of *P. aeruginosa* PAO1. When required, the antibiotics kanamycin (kanamycin sulfate from *Streptomyces kanamyceticus*, BioReagent; Sigma-Aldrich, purity > 95%), streptomycin (Streptomycin sulfate salt, BioReagent; Sigma-Aldrich, purity > 96%), and tetracycline (BioReagent; Sigma-Aldrich, purity > 96%) were incorporated into the growth medium at a concentration of 30 µg/mL or 200 µg/mL in the case of kanamycin, 20 µg/mL for streptomycin, and 100 µg/mL for tetracycline.

Activity of compounds on *Pseudomonas aeruginosa* PAO1 biofilm formation

To determine the MICs of each diarylheptanoid against *P. aeruginosa* PAO1, a standard broth microdilution method was used in LB broth [48]. Tested compounds were included in concentrations from 0.9 µg/mL to 500 µg/mL. The carrier solvent DMSO was used as a control. Cell growth was measured via optical density at 600 nm ($OD_{600\text{ nm}}$) using a Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd.) plate reader after 24 h incubation at 37°C.

P. aeruginosa biofilms were grown in TSB medium without shaking conditions. The effect of diarylheptanoids on biofilm forming ability was tested at concentrations of 50% of the determined MIC (0.5 MIC) for each compound, on polystyrene flat-bottomed microtiter 96-well plates as previously described, with some modifications [49]. Briefly, the overnight culture of *P. aeruginosa* was diluted in a fresh LB medium to $OD_{600\text{ nm}} = 0.2$ and 100 µL of the diluted culture with the appropriate diarylheptanoid at the

concentration of 0.5 MIC was added to the plate. After incubation for 24 h at 37 °C, the plate was washed twice with sterile water, dried, and stained for 30 min with 0.4% crystal violet in order to determine biofilm formed. Stained cells were washed with sterile water to remove the unbound crystal violet. After drying, 150 μ L of 30% (v/v) acetic acid were added to solubilize the dye. After 10 min, the content of the wells was homogenized and the OD of the samples was measured at 550 nm using a Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd.). The experiment was done in quintuplicate and repeated two times.

Effect of diarylheptanoids on twitching and flagella motility of *Pseudomonas aeruginosa* PAO1

Twitching and motility of *P. aeruginosa* PAO1 was assessed after the growth in the presence or absence of diarylheptanoids (10–15 μ g/mL) and streptomycin (20 μ g/mL). *P. aeruginosa* PAO1 cells were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu/mL ($OD_{600nm} = 0.1$). Cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37 °C. Plates were removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope [40,41]. An assay to test flagella-mediated motility was performed as previously described [40,41]. Test compounds were mixed into 10 mL of molten MH medium and poured immediately over the surface of a solidified LB plate as an overlay. The plate was point inoculated with an overnight culture of *P. aeruginosa* PAO1 once the overlaid agar had solidified and then was incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony [32]. The experiment was done in triplicate and repeated two times. The colony diameters were measured three times in different directions, and values are presented as the mean values \pm SE (standard error).

Effect of diarylheptanoids on pyocyanin synthesis in *Pseudomonas aeruginosa* PAO1

Overnight culture of *P. aeruginosa* PAO1 grown in Kings Medium A (Himedia) was diluted to $OD_{600nm} = 0.2$ (5 mL). Different diarylheptanoids at a concentration of 50 μ g/mL were added to the flasks with *P. aeruginosa* PAO1 and incubated at 37 °C for 24 h. Cultures were then extracted with chloroform (3 mL) and the chloroform layer was transferred to a fresh tube and mixed with 0.2 M HCl (1 mL) giving it a pink to deep red color, indicating the presence of pyocyanin. To determine the amount of pyocyanin, the absorbance was measured at 520 nm [50]. The experiment was done in triplicate and repeated two times. The values are expressed as a ratio (OD_{520}/OD_{600}) \times 100.

Effect of diarylheptanoids on violacein synthesis in *Chromobacterium violaceum* CV026

C. violaceum CV026, mini-Tn5 mutant, depends on exogenous AHLs for violacein production, was used as an indicator organism to monitor QS inhibition in a disc diffusion assay [37]. Briefly, semisolid LB agar (0.3%, w/v; 5 mL) was seeded with 50 μ L of an overnight culture of *C. violaceum* CV026 supplemented by N-hexanoyl-L-homoserine lactone (Sigma) to a final concentration of 5 μ M and poured over the surface of LB agar plates. When the overlaid agar had solidified, sterilized discs containing 200 μ g of each compound and erythromycin (50 μ g) as positive control (Sigma-Aldrich; Bioreagent, purity >95%) were placed on the plates. Petri dishes were incubated in the upright position overnight (30 °C) and examined for violacein synthesis. Inhibition of

violacein synthesis was defined by the presence of white haloes in a purple background.

Assays for acyl homoserine lactones production

For the extraction of AHLs, *P. aeruginosa* PAO1 was grown in LB broth at 37 °C for 24 h and supplemented with the appropriate test compound to a final concentration of 50 μ g/mL from the DMSO stock solution. The control containing the equivalent amount of DMSO was also included. After 24 h of growth, the optical density of the culture at 540 nm (OD_{540nm}) was determined and the cells were centrifuged. Supernatants (20 mL) from these cultures were extracted with the same volume of acidified ethyl acetate [acetic acid, 0.1% (v/v)] for 10 min with shaking and centrifuged to separate the aqueous and ethylacetate phases. The ethylacetate phase was transferred to a clean bottle and the solvent was removed under reduced pressure. The remaining residue was weighted and dissolved in the same solvent in an appropriate amount that corresponded to the following ratio: 1 μ L of final extract corresponding to 1×10^9 cells of the original culture [51]. Experiments were performed in triplicate. Concentrated extracts were used in the AHL assays. Semisolid LB agar (0.3%, w/v; 5 mL) was seeded with 50 μ L of an overnight culture of *C. violaceum* CV026 and poured over the surface of LB agar plates. When the overlaid agar had solidified, sterilized discs containing 10 μ L of each AHL extract were placed on the surface of the plates. These petri dishes were incubated in the upright position overnight (30 °C) and examined for the stimulation of violacein synthesis. QS induction was detected as purple pigmentation of the bioreporter strain grown around the discs. Measurements were made from the outer edge of the discs to the edge of the zones of QS stimulation or QS inhibition. The assays were performed in triplicate.

For the micro-volumetric determination of long-chain (3-oxo-C12-HSL) and short-chain AHL (C4-HSL) levels in *P. aeruginosa* PAO1 culture supernatants, cultures were grown overnight at 37 °C in LB. Cultures were diluted 1 : 1000 in 10 mL LB in the presence of diarylheptanoids or DMSO and grown at 37 °C. After 6 h of growth, the OD_{600} was measured, and 2 mL of culture were centrifuged and the supernatant was filtered and stored at -20 °C until used. An aliquot of culture supernatants (20 μ L) was added to 180 μ L of LB inoculated with *P. aeruginosa* PA14-R3 (measurements of 3OC12-HSL) or *P. aeruginosa* PAOJP2 (measurements of C4-HSL; final $OD_{600} = 0.045$) [52,53]. Plates were incubated at 37 °C with shaking, and cell density (OD_{600}) and bioluminescence (light counts per second, LCPS) were simultaneously measured after 4 h of incubation using a Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd.). Luminescence values were normalized per cell density.

2-Alkyl-4-quinolones production assay

For the detection and quantification of QS molecules 2-alkyl-4-quinolones (AHQs), such as 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ), which represent the major *P. aeruginosa* AHQ signal molecules present in bacterial culture supernatants, a *lux*-based *P. aeruginosa* AHQ sensor was employed in a liquid microtiter plate assay. A PAO1 Δ pqsA mini-CTX *luxPpqsA* biosensor was used for the detection and quantification of AHQ molecules extracted from *P. aeruginosa* PAO1 cultures as described previously [54].

Antiproliferative assay

Antiproliferative activities of diarylheptanoids were measured using the methods described previously [55]. MRC5 cells (human lung fibroblast, obtained from ATCC) were plated in a 96-well flat-bottom plate at a concentration of 1×10^4 cells per well grown in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C, and maintained as monolayer cultures in RPMI-1640 medium supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% (v/v) FBS. After 24 h of MRC5 cells incubation, the media containing increasing concentrations of each tested diarylheptanoid (1, 10, 50, 100, and 250 µg/mL) were added to the cells. Control cultures received the solvent DMSO, and blank wells contained 200 µL of growth medium. As a positive control, doxorubicin hydrochloride (Sigma-Aldrich, 98–100% purity by HPLC) was used because it is a commonly used chemotherapeutic drug for the treatment of acute leukemia, lymphomas, and different types of solid tumors such as breast, liver, and lung cancers. After 48 h of incubation, cell proliferation was determined using the MTT reduction assay. Cell proliferation was determined from the absorbance at 540 nm on a Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd.). The MTT assay was performed two times in four replicates and the results are presented as the percentage of the control (untreated cells) that was arbitrarily set to 100%.

Statistical analysis

For each species, three samples were used and all of the assays were carried out in triplicate. The results are expressed as mean values and standard errors, and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 18.0 program.

Supporting information

Minimal inhibitory concentrations (MIC, µg/mL) of diarylheptanoids against *P. aeruginosa* planktonically grown cells, activity of diarylheptanoids on twitching and motility of *P. aeruginosa* PAO1 are available as Supporting Information.

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Conflict of Interest

▼
The authors declare no conflict of interest.

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