

## Antibacterial and quorum sensing regulatory activities of some traditional Eastern-European medicinal plants

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The objective of this study was to screen extracts of twenty Eastern European medicinal plants, using wild-type and reporter *Chromobacterium violaceum* bioassays, for novel components that target bacterial cells and their quorum sensing (QS) communication systems. Three types of activity and their combinations were revealed: (i) direct antimicrobial growth-inhibitory activity, (ii) non-specific and specific pro-QS activities, (iii) anti-QS activity. Among seven plant extracts showing direct growth-inhibitory activity, the strongest effect was shown by *Arctostaphylos uva-ursi* (bearberry) leaves. Many plants stimulated violacein production by wild-type *C. violaceum* ATCC 31532 in a non-specific manner, and only the herb *Bidens tripartita* (three-lobed beggarticks) contained compounds that mimic acyl-homoserine lactone and operated as a QS agonist. Anti-QS activity was found in eleven plants including *Quercus robur* (oak) cortex, *Betula verrucosa* (birch) buds and *Eucalyptus viminalis* (Manna Gum) leaves. Subsequent statistical analysis showed differences between antimicrobial and anti-QS activities, whereas both activities were defined by phylogenetic position of medical resource plant. Finally, extract from *Quercus robur* cortex revealed at least two fractions, showing different anti-QS mechanisms. These data confirm that multicomponent anti-infectious mechanisms are used by plants, which may be useful for drug development.

**Keywords:** medicinal plants, antimicrobial activity, quorum sensing, *Chromobacterium violaceum* bioassay

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The increasing resistance of human and animal bacterial pathogens to a wide spectrum of antibiotics has become an emerging problem in the 21st century (1). In view of the slow progress in the development of antibiotic classes that can overcome drug-resistance mechanisms, the next generation of safe and effective antimicrobial drugs is likely to be

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based on novel sources and strategies (2). In particular, medicinal plants used by traditional (folk) medicine for infection control might represent a rich bio-resource of genuine antimicrobial compounds acting upon novel microbial targets (3).

Early studies of medicinal plant drug discovery focused mainly on their direct antimicrobial potential (4), which has led to investigation of their mechanisms of action and to isolation of diverse active compounds (5). However, much less attention has been paid to alternative medicinal plant anti-infection properties that act on microbes by targeting cell-to-cell communication, also known as quorum sensing (QS) (6). This phenomenon is mediated *via* small signaling molecules (autoinducers) constantly produced and released by bacteria, which upon reaching a critical threshold concentration, bind to receptors and regulate the expression of target genes (7). Although there are a number of different QS paradigms, the most widely used by Gram-negative bacteria is the LuxI/R system, where the LuxI proteins synthesise the signal molecule acyl-homoserine lactone (AHL), and the LuxR receptor proteins induce the expression of QS-target genes following AHL binding (8). In this context, it is important that QS activates virulence factor genes (9) and also that LuxI/R systems in human, animal and plant pathogens are similar (10). Thus, the discovery of QS represents a novel target for antimicrobial drugs and potential plant-produced QS-regulating agents directed against plant-associated bacterial communication systems might provide a novel approach to controlling bacterial infection in humans and animals (11).

The first plant-produced QS inhibitor was isolated from a red macroalga, *Delisea pulchra*, in which halogenated furanones block AHLs *via* competitive inhibition and destabilisation of the LuxR receptor (12). Recently, anti-QS properties have also been identified in terrestrial plants from southern Florida (13) and India (14) and also in herbs used in traditional Chinese (15) and oriental (16) medicine.

The objective of this study was to screen Eastern-European medicinal plants for their direct (growth inhibition) and indirect (QS-regulated) activities. Antibacterial potential was analysed by examining aqueous and ethanol plant extracts using *Chromobacterium violaceum*, a Gram-negative facultatively anaerobic beta-proteobacterium, which is abundant in soil and water ecosystems and occasionally becomes an aggressive opportunistic pathogen (17). Similarly to other clinically significant multidrug-resistant microorganisms, *C. violaceum* shows resistance against numerous antibiotics and drug efflux pump genes (18) and thus represents a model for antibacterial screening. *C. violaceum* also uses a LuxR/I-type QS system (19), where CviI is an *N*-hexanoyl-L-homoserine lactone (C<sub>6</sub>-AHL) synthase, and CviR is a cytoplasmic receptor protein (DNA) binding transcription factor that activates gene expression following binding to a diffusible autoinducer C<sub>6</sub>-AHL (20). As the purple pigment violacein is under the control of this system, the inhibition of pigment production indicates the presence of anti-QS activity; therefore, *C. violaceum* bioassays are suitable for investigations of this type (13–17).

## EXPERIMENTAL

### *Plant material and extract preparation*

Different parts of twenty herbs, flowers and trees were collected for antibacterial and quorum sensing-regulating activities screening (Table I). The plants were selected based on their efficacy in Eastern-European traditional medicine as antiinflammatory drugs

Table I. Traditional Eastern-European medicinal plants screened for antibacterial and quorum sensing-regulating activities

Plant species (family)	Common name	Plant part	Traditional medicinal use
<i>Achillea millefolium</i> (Asteraceae)	Yarrow	Herb	Powerful »healing herb« used topically for wounds, cuts and abrasions as a diaphoretic, astringent, analgesic and head cold remedy
<i>Arctostaphylos uva-ursi</i> (Ericaceae)	Bearberry	Leaves	Antimicrobial and a mild diuretic, used for urinary tract complaints, including cystitis and urolithiasis
<i>Betula verrucosa</i> (Betulaceae)	Birch	Buds	Antimicrobial and diuretic, used for treatment of kidney stones, rheumatism and gout
<i>Bidens tripartita</i> (Asteraceae)	Three-lobed beggarticks	Herb	Antiinflammatory and wound-healing properties, for treatment of diseases of the liver, spleen, bronchitis, diabetes, poor digestion, colds, as a diuretic and diaphoretic, improves digestion
<i>Calendula officinalis</i> (Asteraceae)	Marigold	Flowers	Antiinflammatory, antiviral, antigenotoxic, antiinflammatory, controls bleeding, and soothes irritated tissue, effective in treating radiation dermatitis, abdominal cramps and constipation
<i>Chelidonium majus</i> (Papaveraceae)	Greater celandine	Herb	Antimicrobial, mild analgesic, cholagogic and central nervous system sedative, for removal of warts, used in the treatment of gallstones and dyspepsia
<i>Comarum palustre</i> (Rosaceae)	Marsh cinquefoil	Rhizome	Wound-healing and analgetic remedy for treatment of lung and joint diseases, dysentery and stomach cramps
<i>Eucalyptus viminalis</i> (Myrtaceae)	Manna Gum	Leaves	Antiseptic for deodorising and treatment of cold
<i>Inula helenium</i> (Asteraceae)	Inula Helen	Rhizome	Antimicrobial, antifungal, antitussive, antiparasitic, antiseptic and diuretic properties, for treatment of cough, cold, asthma and bronchitis
<i>Juniperus communis</i> (Cupressaceae)	Common juniper	Fruit	Strong urinary tract disinfectant, treatment of skin infections and diabetes
<i>Ledum palustre</i> (Ericaceae)	Wild rosemary	Propagules	Antiinflammatory and antispasmodic properties, for treatment of cough, lung and urinary disorders
<i>Matricaria chamomilla</i> (Asteraceae)	Chamomile	Flowers	Antimicrobial and antiinflammatory, mild laxative, as a mouthwash, for treatment of sore stomach, diarrhoea

Table I. Continued

<i>Plantago major</i> ( <i>Plantaginaceae</i> )	Plantain	Leaves	Weak antimicrobial, antiinflammatory, analgesic, antioxidant, immunomodulating activity, to treat wounds, fever, respiratory infections, diarrhoea or dysentery
<i>Quercus robur</i> ( <i>Fagaceae</i> )	Oak	Rind	Antimicrobial and antiinflammatory, as astringent, for treatment of inflammation and diarrhoea
<i>Rosa majalis</i> ( <i>Rosales</i> )	Briar	Fruit	Antimicrobial and antifungal, as a source of vitamin C
<i>Salvia officinalis</i> ( <i>Lamiaceae</i> )	Sage	Leaves	Antimicrobial, pain killer, for treatment of colds, cardiovascular and cerebrovascular diseases
<i>Taraxacum officinale</i> ( <i>Asteraceae</i> )	Dandelion	Rhizome	Antimicrobial and antiviral, to treat infections, bile and liver problems, for increasing appetite and for improving digestion, as a diuretic, mild laxative
<i>Tussilago farfara</i> ( <i>Asteraceae</i> )	Coltsfoot	Leaves	Treatment of bronchitis, asthma, whooping cough and for treatment upper respiratory tract complaints including sore mouth and throat, cough, and hoarseness
<i>Vaccinium vitis-idaea</i> ( <i>Ericaceae</i> )	Lingonberry	Leaves	Disinfectant/antiseptic, depurative, astringent, antihemorrhagic, diuretic, and for treatment of diabetes mellitus, rheumatism
<i>Viola tricolor</i> ( <i>Violaceae</i> )	Heartsease	Herb	Antiinflammatory, for treatment of skin diseases and eczema, epilepsy, respiratory problems: bronchitis, asthma and cold symptoms, also a diuretic, leading to its use in treating rheumatism and cystitis

and cures for infection-associated diseases (skin lesions, wounds, respiratory disorders, diarrhoea, *etc.*) according to the instructions accompanying commercially available preparations. They were purchased from Russian ethnopharmaceutical companies as dried leaves, flowers, fruits, rhizomes, buds, propagules and cortex material. The plant material was ground to a fine powder using an electric mill and extracted using water or ethanol. For aqueous extracts, 6 g of each sample was added to 50 mL of hot sterile distilled water. Depending on whether the tissue was soft or tough, this was boiled for 15 min (leaves, flowers), or for 30 min (rhizomes, cortex) and cooled at room temperature for 10–15 min. Primary aqueous extracts were centrifuged at 1000×g for 10 min to remove particulate matter; the supernatants were collected and filtered through a 0.2-µm pore size polyethersulfone (PES) syringe filter (Membrane Solutions LLC, USA) into autoclaved vials to ensure sterility of the final samples. For ethanolic extracts, the plant powder was added to 45 % (V/V) ethanol (3 g dry mass per 25 mL), allowed to stand for 24 h at room temperature before centrifugation and upper phase filtration as above. Final samples were stored as 1-mL aliquots at +4 °C until further analysis.

### *Separation of bioactive fractions from plant extracts*

The rotary evaporated dried plant extracts (5 mg) were dissolved in 25 mL of 0.1 % trifluoroacetic acid (TFA) and applied to a Synchrorep RP-P C8 phase (SynChrom Inc., USA) 5-mL column. After elution of all un-bound compounds, a total hydrophobic fraction was eluted with 65 % acetonitrile in water by the addition of 0.1 % TFA. Absorbance was monitored at 280 nm at a flow rate of about 2 mL min<sup>-1</sup>. Acetonitrile was evaporated in a Speedvac vacuum centrifuge (Savant, USA). The fractions were lyophilized (Labconco, USA) and then dissolved in sterile distilled water for subsequent bioactivity analyses.

### *Bacterial strains and culture conditions*

The wild-type strain *C. violaceum* ATCC 31532 was purchased from the American Type Culture Collection (LGC Standards, UK). This strain uses a LuxI/R-type quorum sensing system (20) that produces and responds to the cognate autoinducer C<sub>6</sub>-AHL, positively controlling a *vio*ABCDE-operon (21). Expressed at high cell density, VioA-E proteins perform enzymatic oxidation and coupling of two molecules of tryptophan to give a rearranged pyrrolidone-containing scaffold in the resulting purple pigment violacein (22). The other biomonitor strain, *C. violaceum* NCTC 13274, was acquired from the National Collection of Type Cultures (Health Protection Agency, UK). This mini-Tn5 mutant of the wild-type strain has an insertion in the *cvil* gene and is unable to produce its own autoinducer but responds to exogenous active signal molecules and produces the characteristic purple pigment violacein following incubation with C<sub>6</sub>-AHL. Thus, the wild-type strain recognises anti-QS compounds, including inhibitors of AHL signal generation and reception, whereas the mutant (reporter) strain is reactive to the inhibition of AHL signal reception only, which in both cases leads to inhibition of violacein production and makes these strains ideally suitable for anti-QS activity screening (23).

Both bacterial strains were stored as 40 % glycerol stocks at -20 °C and were cultured in Luria-Bertani (LB) broth medium (AppliChem GmbH, Germany) with 10 mg mL<sup>-1</sup> glucose at 27 °C prior to use.

### *Bacterial growth inhibition and violacein production screening assay*

The integral agar-diffusion assay was used to detect both antimicrobial and anti-QS activity of the medicinal plant extracts by means of double layer culture plates. Briefly, 5 mL of warm molten 0.5 % LB agar was inoculated with 100 µL of freshly prepared *C. violaceum* ATCC 31532 or *C. violaceum* NCTC 13274 culture. Mutant strain was used in two modifications: without exogenous signal or supplemented with 5×10<sup>-7</sup> mol L<sup>-1</sup> C<sub>6</sub>-AHL (Cayman Chemical Company, USA). Agar and inoculum were gently mixed and placed immediately over the Petri dish, which was previously overlaid with layer (10 mL) of 1.5 % LB-agar. Solidified agar plates were punched with a 5-mm diameter flame-sterilised cork borer and were kept undisturbed for 30 min for absorption of excess moisture. The wells were filled with 30 µL of appropriate aqueous or ethanolic plant extracts with an equal amount of either sterile distilled water or 45 % ethanol used as a negative control. In some experiments, plant extract mixtures and separated compounds were tested. The assay plates were incubated overnight at 27 °C for 24–48 h and were then examined for culture growth and violacein production.

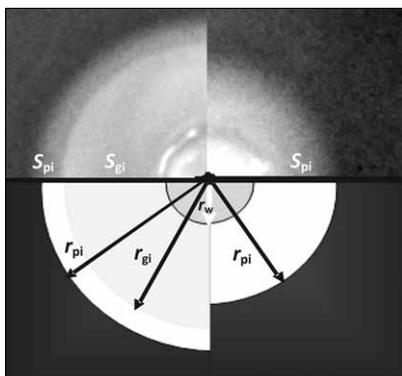


Fig. 1. Quantitation of antimicrobial and anti-QS activity in an integral agar-diffusion *C. violaceum* assay.

Direct antimicrobial activity was determined as the growth inhibition (*gi*) area around wells and was calculated as  $S_{gi} = \pi r_{gi}^2 - \pi r_w^2$ , where  $r_{gi}$  is the radius (mm) of the area devoid of bacterial growth and  $r_w$  is the well radius (mm). The anti-QS activity was detected by pigment inhibition (*pi*) as a colorless but viable halo on a purple background around the growth inhibition zone and was calculated as  $S_{pi} = \pi r_{pi}^2 - \pi r_{gi}^2 - \pi r_w^2$  or as a colorless halo around the well without growth inhibition and calculated as  $S_{pi} = \pi r_{pi}^2 - \pi r_w^2$  (Fig. 1).

## RESULTS AND DISCUSSION

### *Antibacterial activity of plant extracts against C. violaceum*

To validate the traditional ethnopharmacological uses of the tested plants as direct antibacterial agents, aqueous and ethanolic extracts were applied in the radial growth inhibition assay against wild-type *C. violaceum* ATCC 31532 and reporter *C. violaceum* NCTC 13274 strains (Table II).

Among the plant extracts that showed direct growth inhibitory activity, the strongest effect was shown by *Arctostaphylos uva-ursi* (bearberry) leaves against *C. violaceum* ATCC 31532 (clear zone area of 235 mm<sup>2</sup>) and *C. violaceum* NCTC 13274 (207 mm<sup>2</sup>). The antibacterial activity of *Arctostaphylos uva-ursi* against *Pseudomonas aeruginosa* was recently revealed (24), and according to the data presented, this medicinal plant, traditionally used as an antimicrobial for urinary tract complaints including cystitis and urolithiasis, can be recommended as an antibacterial agent against both *C. violaceum* and *P. aeruginosa*, which cause severe and often fatal infections in humans and animals (25).

The diffusion test results showed the prominent growth-inhibition activity of aqueous extracts of *Vaccinium vitis-idaea*, *Eucalyptus viminalis* and *Chelidonium majus* (clear zones areas 31, 19 and 9 mm<sup>2</sup>, respectively). Further, in the growth inhibition assay, the ethanolic extracts showed a more pronounced activity, which led to the enlargement of

Table II. Anti-microbial and anti-QS activity of the screened aqueous and ethanolic plant extracts against wild-type *C. violaceum* ATCC 31532 and reporter *C. violaceum* NCTC 13274 strain

Plant species	Inhibition zone area (mm <sup>2</sup> ) against wild-type <i>C. violaceum</i> ATCC 31532 strain			Inhibition zone areas (mm <sup>2</sup> ) against reporter <i>C. violaceum</i> ACTC 13274 strain <sup>a</sup>		
	Growth inhibition		Pigment inhibition	Growth inhibition		Pigment inhibition
	Aqueous extract	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract	Ethanolic extract
<i>Arctostaphylos uva-ursi</i>	235	207	126	189	207	235
<i>Betula verrucosa</i>	0	19	89	105	0	9
<i>Calendula officinalis</i>	0	0	0	19	0	0
<i>Chelidonium majus</i>	9	19	0	0	9	14
<i>Comarum palustre</i>	0	0	0	19	0	0
<i>Eucalyptus viminalis</i>	19	31	75	127	19	31
<i>Inula helenium</i>	0	0	0	35	0	0
<i>Juniperus communis</i>	0	0	31	31	0	0
<i>Ledum palustre</i>	0	31	0	19	0	19
<i>Quercus robur</i>	0	0	75	94	0	0
<i>Rosa majalis</i>	0	0	0	9	0	0
<i>Salvia officinalis</i>	0	31	0	0	0	25
<i>Vaccinium vitis-idaea</i>	31	44	0	69	31	59
					276	153
					113	105
					0	31
					0	0
					0	75
					276	204
					0	85
					0	19
					19	25
					94	94
					0	14
					0	0
					83	54

<sup>a</sup> Supplemented with C<sub>6</sub>-AHL (5 × 10<sup>-7</sup> mol L<sup>-1</sup>).

*Achillea millefolium*, *Bidens tripartita*, *Matricaria chamomilla*, *Plantago major*, *Taraxacum officinale*, *Tussilago farfara* and *Viola tricolor* did not show any activity.

growth inhibition zones in comparison with aqueous extracts (except for *Arctostaphylos uva-ursi*), and also the appearance of clear areas around wells filled with ethanolic extracts of *Salvia officinalis* (25–31 mm<sup>2</sup>), *Ledum palustre* (19–31 mm<sup>2</sup>) and *Betula verrucosa* (9–19 mm<sup>2</sup>), with no effect around control wells filled with 45 % ethanol. In turn, the wild-type *C. violaceum* strain was slightly more sensitive than the reporter and was more inhibited by ethanolic plant extracts than aqueous extracts.

Correlation analysis of growth inhibition rates showed a very high degree of conformity between the antibacterial activity of aqueous ( $R = 0.998$ ,  $p < 0.001$ ) and ethanolic plant extracts ( $R = 0.994$ ,  $p < 0.001$ ). These extracts showed similar activity against both wild-type and mutant *C. violaceum* strains; significant correlation coefficients showed equal sensitivity of *C. violaceum* ATCC 31532 and *C. violaceum* NCTC 13274 to the extracts of the same plant. Thus, the initial screening of twenty species of Eastern-European medicinal plants showed direct antimicrobial activity for seven of them, which explains their traditional use as cures for inflammations and infection-associated diseases. The majority of plant extracts, however, had no effect on the growth of the tested microorganisms, indicating the presence of indirect antimicrobial effects, such as QS-regulatory activity.

#### *Regulatory effects of plant extracts on C. violaceum pigment production*

The integral agar-diffusion assay exhibited several effects of plant extracts on violacein production, which were not completely identical in wild-type and reporter *C. violaceum* biotests.

Surprisingly, many plants stimulated violacein production by the wild-type *C. violaceum* ATCC 31532 strain and showed an intensive purple halo around wells filled with aqueous or ethanolic extracts against a light purple background. To investigate the nature of this effect in the presence of AHL-like compounds that mimic the native bacterial auto-inducer, plant extracts were tested against the *C. violaceum* NCTC 13274 reporter, without C<sub>6</sub>-AHL supplementation. Among twenty species of Eastern-European medicinal plants, only aqueous and ethanolic extracts of *Bidens tripartita* (three-lobe beggarticks) induced a concentric purple halo. This finding may hint at the conjecture of some plants being producers of specific compounds that mimic bacterial signal activities and operate as QS agonists (26); this especially holds for plant-bacteria symbiotic interactions. However, our experience of screening a multitude of extracts has provided the impression that an increase in the violacein content was more often caused by unknown non-specific mechanisms, possibly *via* the addition of nutritional substrates, which led to more rapid increases in cell density and the development of QS communication. Furthermore, the stimulation of pigment production by *C. violaceum* NCTC 13274 supplemented with C<sub>6</sub>-AHL was reduced, probably because of the initially high autoinducer levels in the cultivation environment.

Subsequent analysis revealed various degrees of violacein inhibition, resulting in a viable but colorless zone, by 11 of the 20 screened plants (Table II), which showed anti-QS activity more ubiquitously than direct antibacterial activity. Here, we report for the first time the prominent anti-QS properties of *Betula verrucosa* buds (non-pigmented zone area of 89–113 mm<sup>2</sup>), while direct antibacterial properties of birch extracts were shown earlier (27). We are also reporting the anti-QS activity of *Eucalyptus viminalis* leaves (75–276 mm<sup>2</sup>), which agree well with data on the anti-QS properties of *Eucalyptus* oil

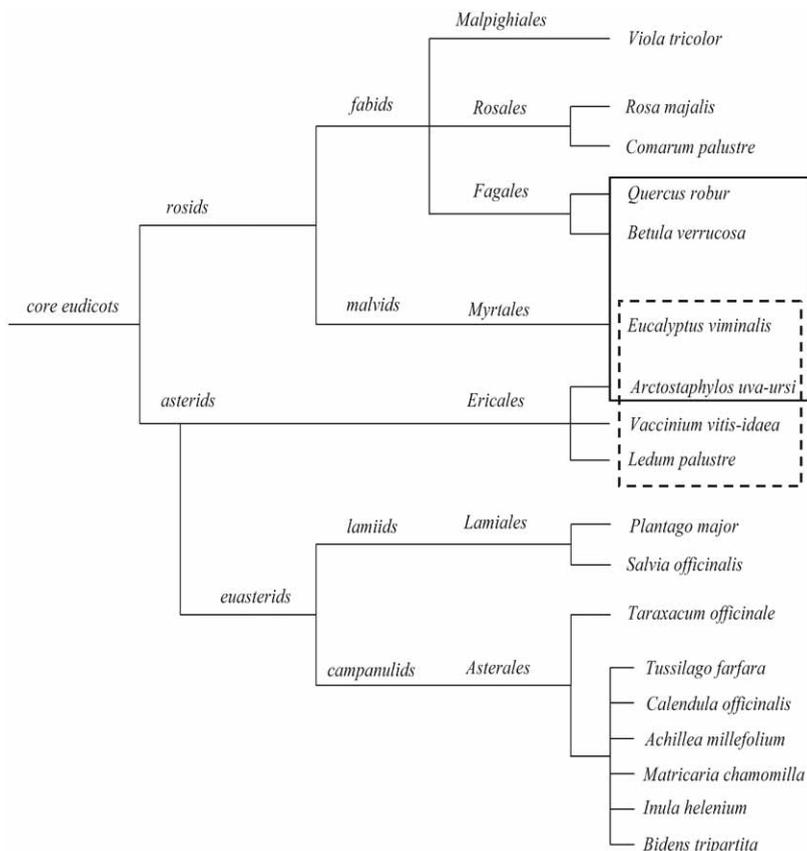


Fig. 2. Based on APG III (31), a tree was prepared showing the phylogenetic relationships of the medicinal plants used in this study, including prominent antibacterial (in dashed frame) and anti-QS (in solid frame) plants. *Juniperus communis* (division *Pinophyta*) and *Chelidonium majus* are not shown.

(28) as well as a significant reduction in QS-mediated biofilm formation by *Eucalyptus globulus* extracts (29). Finally, we have confirmed the anti-QS activity of *Quercus robur* cortex (74–94 mm<sup>2</sup>) previously described by Adonizio *et al.* (30) in *Quercus virginiana*.

Loss of violacein content was shown on the border of growth inhibition zones around wells filled with aqueous and ethanolic extracts of *Arctostaphylos uva-ursi* leaves (non-pigmented zone area 126–189 mm<sup>2</sup>). These data agree with the findings showing that *Arctostaphylos uva-ursi* decreases the QS-mediated elastolytic and proteolytic properties of *P. aeruginosa* without effects on pyocyanin and pyoverdine formation (24), which is complementary to the direct antimicrobial effect of this medicinal plant (see above).

Aqueous extracts of *Vaccinium vitis-idaea* and *Ledum palustre* showed a decrease in the C<sub>6</sub>-AHL-mediated violacein content in the reporter strain *C. violaceum* NCTC 13274 (non-pigmented zone areas 83 and 19 mm<sup>2</sup>, respectively), but were inactive against the wild-type strain where a 31-mm<sup>2</sup> violacein inhibition zone was induced by the *Juniperus*

*communis* aqueous extract. Ethanolic extracts of these medical plants were able to reduce pigmentation in both *C. violaceum* biotests. Further, as shown in Table II, noticeable violacein inhibition was only observed with ethanolic but not with aqueous extracts of *Inula helenium*, *Calendula officinalis*, *Comarum palustre* and *Rosa majalis*. Thus, we have confirmed that the type of extractant plays a role for the level of medicinal plant bioactivity (29). This explains the use of ethanolic extracts in Eastern European (in particular, Russian) ethnomedicine.

Correlation analysis of violacein inhibition data showed more significant correlations for *C. violaceum* ATCC 31532 ( $R = 0.926$ ,  $p < 0.001$ ) and *C. violaceum* NCTC 13274 ( $R = 0.904$ ,  $p < 0.001$ ) strains treated with aqueous or ethanolic plant extracts than comparative effects of aqueous ( $R = 0.749$ ,  $p < 0.01$ ) or ethanolic ( $R = 0.790$ ,  $p < 0.01$ ) extracts against both strains. Hence, the results demonstrate the complementary character of wild-type and reporter *C. violaceum* biotests and reveal the total anti-QS properties of the screened medicinal plants.

### *Comparative antibacterial and regulatory activities of plant extracts*

As several plant extracts showed both antibacterial and anti-QS activities, the statistical relationship among them was analysed. The Pearson correlation coefficient for *C. violaceum* ATCC 31532 and *C. violaceum* NCTC 13274 strains treated with aqueous or ethanolic plant extracts was calculated, and the results of two random variables were shown as numerical values and linear dependences for data sets. The correlation between antibacterial ( $S_{gi}$  – area of growth inhibition, mm<sup>2</sup>) and anti-QS ( $S_{pi}$  – area of pigment inhibition, mm<sup>2</sup>) variables for the *C. violaceum* ATCC 31532 strain was statistically significant. Correlation coefficients for aqueous and ethanolic extracts were  $R = 0.642$  and  $R = 0.706$  ( $p < 0.01$ ), respectively, *i.e.* both extracts types showed both activities in the wild-type biotest. However, no accurate linear relationship could be established between the two variables. This points to the probable simultaneous presence of both antibacterial and anti-QS activities in plant extracts, but also indicates the absence of their strong interdependence. Also, in *C. violaceum* NCTC 13274 reporter strain, the statistical relationships between antibacterial and anti-QS variables became statistically insignificant. Correlation coefficients for the interrelationships between these parameters were  $R = 0.430$  for aqueous and  $R = 0.358$  for ethanolic extracts and could not be linearized correctly. The data thus demonstrate differences between antibacterial and anti-QS plant compounds; they function as growth inhibitors or as receptors of AHL signals, respectively.

In addition, we performed comparative analyses of antibacterial and anti-QS activities of medicinal plants according to the Angiosperm Phylogeny Group (31) classification. We have shown that predominantly antibacterial and anti-QS plants were located on different branches of the phylogenetic tree of *core eudicots* and were united in two partially overlapping groups (Fig. 2). The most prominent antibacterial plants were put into the *asterid* clade, order *Ericales*, indicating the similarity of their evolutionarily developed anti-infection strategy *via* direct suppression of pathogen growth. In turn, the prominent anti-QS plants were put into the *rosid* clade with the closest ties between the *Quercus* and *Betulla* genera, both related to the order *Fagales*. *Eucalyptus viminalis* (*rosid* clade, order *Myrtales*) combined these activities as both antibacterial and anti-QS medicinal plant.

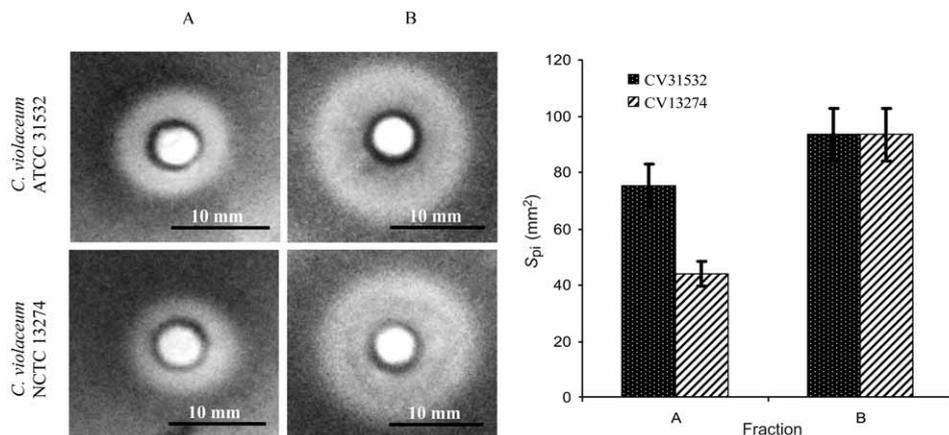


Fig. 3. Quantitative analysis of violacein inhibition in wild-type *C. violaceum* ATCC 31532 and reporter *C. violaceum* NCTC 13274 bioassays by fractions A and B of the *Quercus robur* cortex extract. Images (left) show non-pigmented zones and the data are represented as violacein inhibition zone areas ( $S_{pi}$ ) in  $mm^2$  (right). Mean values of triplicate independent experiments and SDs are shown.

These data thus confirm the differences between predominant antibacterial and anti-QS properties in various source plants. Therefore, the dependence of these bioactivities on plant phylogeny provides a basis for further bioinformatic assessments of antibacterial and regulatory compounds.

#### *Anti-QS fractions isolated from Quercus robur cortex extract*

The *Quercus robur* cortex that showed prominent anti-QS activity without detectable antibacterial activity was chosen for the analysis of bioactive compounds.

Chromatographic separation of the dried extract was performed by means of one-step liquid chromatography on a reversed-phase column under low pressure conditions. Separation of total extracts enabled us to obtain several mixed fractions characterized by the presence of hydrophobic (fraction A) and hydrophilic (fraction B) components. Our preliminary data allowed us to conclude that fraction A predominately consisted of basic proteins/peptides, glycoproteins/glycopeptides, some polyphenols (pigments, kinones, *etc.*), while fraction B contained hydrophilic low molecular mass organic compounds.

Analysis of the bioactivity of fractions A and B showed differences in the anti-QS effect against wild-type and reporter *C. violaceum* strains (Fig. 3). The A fraction caused a two-fold higher inhibition of violacein production by the wild-type *C. violaceum* ATCC 31532 strain (non-pigmented zone area  $75\text{ mm}^2$ ) compared to the *C. violaceum* NCTC 13274 reporter strain ( $44\text{ mm}^2$ ), which that acts not only *via* signal reception but also by suppression of AHL biosynthesis. In turn, fraction B showed a more pronounced effect in both *C. violaceum* ATCC 31532 and *C. violaceum* NCTC 13274 biotests (non-pigmented zone area  $94\text{ mm}^2$  in both cases), which suggested the presence of an anti-QS compound

as a probable inhibitor of AHL signal reception. These results show that the anti-QS activity in plant extracts might be driven by two or more compounds representing different bioactivity mechanisms.

## CONCLUSIONS

Use of plant extracts is common in traditional (folk) medicine for the treatment of infection and inflammation and has stimulated scientific interest in screening programmes for novel bioactive components. Plant drug discovery has been focused on the prevention of microbial growth and direct killing of bacterial pathogens, or against an alternative anti-infective mechanism that uses cell-cell signalling mediated through quorum sensing. To the best of our knowledge, no studies that would evaluate both activities and address their interactions have been performed. Thus, in this study, some Eastern-European medicinal plants were screened for growth inhibition and QS-regulating activities using two *C. violaceum* strains: wild-type ATCC 31532 and the derived reporter NCTC 13274, which are ideally suited for combined antibacterial and anti-QS activity screening.

Three types of activities and their combinations were shown in 14 of the 20 tested plants: direct antimicrobial growth-inhibitory activity, non-specific and specific pro-QS activity and anti-QS activity. Analyses have shown the effect of combined antibacterial and anti-QS activities in plant extracts, although statistical analysis has also shown differences between these activities, depending on source plant phylogeny.

In conclusion, these data show multi-component anti-infectious mechanisms used naturally for plant-associated bacterial control and inhibition of phytopatogenic bacteria recognized by folk medicine for the treatment of human and animal infections. Thus, the paradigm »one plant – one component – one effect« should be criticized and further studies on the purification of active components and reproduction of medicinal plant activity for anti-infectious drug development based on novel strategies are necessary.

Some Eastern-European medicinal plants whose activity is reported now are promising sources in the search for microbial growth and quorum sensing inhibitors.

Here, we started the separation procedure of the *Quercus robur* cortex extract, showing prominent anti-QS properties. We are planning to continue experiments to separate the fractions obtained in this study and further identify the structure of active molecules.

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