Evaluation of quorum-sensing inhibitory effects of extracts of three traditional medicine plants with known antibacterial properties

F. Moradi1), N. Hadi1)2) and A. Bazargani1)
1) Department of Bacteriology and Virology, School of Medicine and 2) Bioinformatics and Computational Biology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Today an alternative approach to control bacterial infections is the use of natural and traditional plant compounds to interfere with their quorum-sensing (QS) systems. In this study, antibacterial and anti-QS sensing activity of Syzygium aromaticum, Dionysia revoluta Boiss. and Eucalyptus camaldulensis Dehnh. were evaluated. These plants are local to the Middle East region and have since ancient times been used for their antibacterial activity. Plant compounds were extracted with n-hexane, methanol and 96% ethanol mixed solvent. Antibacterial activity of this herbal extracts against five Gram-negative and Gram-positive bacteria were assessed. The effective sub−minimum inhibitory concentration (MIC) of this extract on bacterial QS systems were investigated by a violacein quantification assay in the Chromobacterium violaceum CV026 biosensor strain, inhibition of exogenously QS signal molecules in Aeromonas veronii bv. Sobria strain BC88 and Pseudomonas aeruginosa isolated from a patient with cystic fibrosis in vitro. Results found that Syzygium aromaticum 0.39 to 0.048 mg/mL, Dionysia revoluta 3.1 to 0.39 mg/mL and E. camaldulensis 0.78 to 0.097 mg/mL showed anti-QS activities by reducing the violacein formation depletion of QS signals produced in A. veronii and P. aeruginosa at sub-MICs. Regarding the anti-QS effects of these herbal extracts, their effective components may be candidates for use in combating bacterial infections at sub-MICs.

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Keywords: Anti-quorum sensing, Dionysia revoluta, Eucalyptus camaldulensis, plant extracts, Syzygium aromaticum

Introduction

Controlling bacterial infections has recently become difficult because of the excessive use of antibiotics in human medicine; such therapy is considered to be a major factor in widespread bacterial resistance, and the formation of biofilm greatly reduces the sensitivity of bacteria to antibacterial agents [1–3]. Today, an alternative approach to control bacterial infections is to interfere with their quorum-sensing (QS) mechanisms by decreasing the production of, diffusing and/or destroying of QS signals, or by inhibiting their receptors by imitating signal structures [4]. In bacterial populations, QS regulates biological behaviour including biofilm formation, bioluminescence and enzyme secretion at a threshold population density through signal molecules [5,6]. Finding new ways to target QS in bacteria is a known strategy to find and develop new antibiotics which might provoke a response within the host defense system, even in cases of low-density bacteria [7,8]. Many eukaryotes, fungi, medicinal plants and fruits and vegetables secrete compounds which can interfere with the QS-regulated gene expression in the invading organism [9–13].

One of the recommended methods to deal with drug-resistant bacteria is the use of natural antimicrobial agents such as natural plant compounds. Medicinal plant extracts can play an important role in combating bacterial infection by producing a diverse antimicrobial compound, such as phenolic, terpenoids, flavanones and quinones, as a result of the similarity of their chemical structure to those of QS signals (acyl-
homoserine lactone, AHL) and their ability to degrade signal receptors [4,14,15]. Antimicrobial activities of herbal medicine against some bacterial species have been reported, especially from extracts and fractions of *Syzygium aromaticum, Dionysia revolta* and *Eucalyptus camaldulensis* [16–19]. Furthermore, some researchers have reported antifungal, anti-inflammatory, anticancer and antioxidant effects of these herbal medicines [18,20–22].

We evaluated the antibacterial effects and the role of sub–minimum inhibitory concentrations (MICs) of these herbal extracts to interfere in the QS system of *Chromobacterium violaceum CV026* biosensor strain, *Aeromonas veronii* bv. Sobria strain BC88 and *Pseudomonas aeruginosa* isolated from a hospitalized patient as clinical samples.

**Materials and methods**

**Collection of plant material and extract preparation** The study was approved by the Shiraz University of Medical Sciences (SUMS) ethics committee (approval IR.SUMS.REC.1397.083). Plant materials were obtained from a local market in Shiraz, Iran. A voucher specimen and the scientific identity of the plant compounds were confirmed at the SUMS herbarium department. A voucher specimen was deposited in the herbarium of the Shiraz Faculty of Pharmacy, Shiraz, Iran. The plant samples were washed twice with distilled water and dried in the dark at 25°C for 72 hours. The dried plant samples were powdered and submerged in 600 mL of n-hexane, methanol and 96% ethanol mixture solvent (ratio 1:1 w/v) in a rotary for 72 hours. The percentage yields of different extracts were calculated using following formula (Table 1):

\[
\text{Yield} (%) = \frac{\text{dry crude extract}}{\text{dry initial plant material before extraction}} \times 100
\]

The plant extracts were filtered and concentrated with a rotary evaporator and solved in appropriate concentrations of dimethyl sulfoxide (DMSO; 50 to 0.048 mg/mL) until further analysis.

**Bacterial strain and culture conditions**

For the antibacterial assays, five clinical isolates—*Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter baumannii, Seratia marcescens* and *Pseudomonas aeruginosa* (isolated from a patient with cystic fibrosis)—were provided from the clinical bacteriology laboratory of Shahid Faghihi Hospital, Shiraz, Iran. *Aeromonas veronii* bv. Sobria BC88, *Chromobacterium violaceum CV026* (mini-Tn5 mutant of the wild-type strain; NCTC 13278), −80°C stock in the Bacteriology and Virology Department of SUMS, were used for the QS inhibition assay. We used C6-AHL (N-hexanoyl-L-homoserine lactone) in this study as a signal molecule (56395-10MG; Sigma-Aldrich, St Louis, MO, USA). *C. violaceum CV026* was cultured in Luria-Bertani (LB) medium (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract) supplemented with kanamycin (30 μg/mL) and chloramphenicol (30 μg/mL) with shaking at 220 rpm at 28°C.

**Antibacterial properties**

In this study, we used the disc-diffusion method based on Murray et al. [27] to determine the antibacterial activity of herbal extracts on bacterial strains. Herbal extracts were dissolved in DMSO (50–0.39 mg/mL), and Müller-Hinton agar plates (Merck, Darmstadt, Germany) were uniformly inoculated with 1 mL of each bacterial suspension (10⁸ CFU/mL). Therefore, herbal extract dilutions (50 μL) were placed onto sterile paper discs (6 mm), set on the bacterial culture plate and incubated at 37°C for 48 hours. Tetracycline (30 μg) and DMSO were used as positive and negative controls respectively. The antibacterial activity of herbal extracts was determined by measuring the diameter of the inhibition zone in millimetres.

**Table 1. Plant compounds extracted**

| Characteristic | Species | Dionysia revolta Boiss. | Eucalyptus camaldulensis Dehnh. | Syzygium aromaticum |
|---------------|---------|------------------------|----------------------------------|--------------------|
| Voucher*      | 1093    | Primulaceae             | Primulaceae                       | 1092               |
| Plant family  |         | Stone bride of Iran     | Myrtaceae                         | Myrtaceae          |
| Common name   |         | Whole plant             | Eucalyptus                        | Dianthus           |
| Tested part   | 88      | n-Hexane, methanol, 96% ethanol | Leaves                           | Stem               |
| Weight (g)    | 3.8     | Brown                   | n-Hexane, methanol, 96% ethanol   | 11.5               |
| Solvents (mixed) |       | Extract colours         | Green                            | Green              |
| Yield (%)     |         | Antimicrobial, anti-inflammatory, anticancer | Antibacterial, antifungal, antioxidant | Dark brown       |
| Clinical use  |         | [Reference]             | [Reference]                       | Antibacterial      |
| Main chemical components | 2-Acetophenone, benzaldehyde, 2- aceetyl phoenol, β-farnesyl alcohol, eugenol, rosofolol, γ-eudesmol, α-hydroxychalcone, other [24] | 1,8-Cineole, p-cymene, p-pinene, terpinen-4- ol, aromadendrin, α-terpineol [25] | Eugenol, β-caryophyllene, vanillin, crategolic acid, kaempferol, rhamnetin, eugenin, ellagic acid, gallic acid, biforin, myricetin, other [26] |

*Voucher specimen was deposited in herbarium of Shiraz Faculty of Pharmacy, Shiraz, Iran.

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Minimum inhibitory concentrations

Before the anti-QS assay, the minimal bactericidal concentration (MBC), MIC and sub-MIC values of herbal extracts were determined using serial macrodilution assays in control and test tubes according to 2015 Clinical and Laboratory Standards Institute guidelines [28]. First A. veronii bv. Sobria BC88, P. aeruginosa isolated from a patient and C. violaceum CV026 were cultivated overnight at 37°C in LB broth. All extracts were initially tested at 25 mg/mL and serially diluted twofold (control and test tube) to 0.048 mg/mL. Each test tube contained 0.5 mL of each concentration and was inoculated with equal volumes of the microbial suspension (10⁶ CFU/mL). The control tubes contained herbal extract concentration and broth medium without bacterial inoculum; this was done to eliminate plant extracts and broth media turbidity so we could more easily follow bacterial growth in the test tube during the spectrophotometric assay at 600 nm. An extract-free tube containing a microbial suspension was used as a positive control. All of the tubes were incubated at 37°C for 24 hours. The presence or absence of bacterial growth in the test tubes, a standard 0.25 μL of the suspensions in each tube was inoculated on 3 mm Müller-Hinton agar and incubated overnight at 37°C.

Violacein quantification assay

To explore the anti-QS potential of sub-MICs of the herbal extracts in a 96-well microplate, assays were performed to examine the inhibition of violacein production in C. violaceum CV026. An overnight culture was prepared of C. violaceum CV026 diluted 1:50 in 4 mL of fresh LB broth, supplemented with 0.25 μg/mL C₆-AHL and different sub-MICs of herbal extracts. In this experiment, we used control wells containing C. violaceum CV026 and LB broth to manage bacterial population and without pigment production (negative control, OD₆₀₀nm = 1) and C. violaceum CV026, AHL signal without herbal extracts to evaluate violacein production in the constant bacterial population (positive control). Microplates were incubated for 24 hours at 28°C in a shaking incubator, and the absorbance of each well was read at 585 and 600 nm for violacein production and bacterial population growth respectively with a Polar Star Omega Microplate reader (BMG Labtech, Ortenberg, Germany) (Fig. 1). The experiment was repeated in triplicate, and the percentage of violacein pigment inhibition compared to control wells was calculated using the following equation [30,31]:

\[
\text{Inhibition} \% = \left[ \frac{(\text{OD}_C - \text{OD}_T)}{\text{OD}_C} \right] \times 100
\]

where OD C is OD₆₀₀nm to track bacterial growth in the positive control tube and OD T is OD₆₀₀nm to estimate bacterial growth in the test tubes. To ensure the presence or absence of bacterial growth in the test tubes, a standard loop of the suspensions in each tube was inoculated on 3 mm Müller-Hinton agar and incubated overnight at 37°C.

![FIG. 1. Violacein quantification assay in 96-well plate. (A1–A3) Chromobacterium violaceum CV026 culture without AHL and herbal extract concentration (negative control). (B1–B3) C. violaceum CV026 culture with AHL and without herbal extract concentration as positive control. (C1–F1) C. violaceum CV026 culture with sub—minimum inhibitory concentration (MIC) of Syzygium aromaticum (0.39, 0.195, 0.097, 0.048 mg/mL). (C2–F2) C. violaceum CV026 culture with sub-MIC of Eucalyptus camaldulensis Dehnh. (0.78, 0.39, 0.195, 0.097 mg/mL). (C3–F3) C. violaceum CV026 culture with sub-MIC of Dionysia revoluta Boiss. (3.1, 1.56, 0.78, 0.39 mg/mL). AHL, acyl-homoserine lactone.](image)
Anti-QS activity through QS signal inhibition

Because QS is a vital system to regulate Aeromonas enterotoxin and Pseudomonas virulence factors, and because it contributes to biofilm formation in both [33–35], in vitro screening for the anti-QS activity of herbal extracts was performed against A. veronii bv. Sobria strain BC88 and P. aeruginosa isolated from a hospitalized patient. The C. violaceum CV026 biosensor strain violacein gene is also under the control of QS. This strain has a mutation in the AHL-producing gene, which results in a lack of pigment production without the presence of an external AHL signal. In this trial, Aeromonas and Pseudomonas produce and diffuse the same AHL QS signals in the LB agar plate which are compatible with the C. violaceum CV026 biosensor. These signals could then be taken by the latter bacterium, letting it produce purple violacein pigment. We hypothesize that the antibacterial activity of herbal extracts can be due to their interference with the QS system of certain bacteria, blocking bacterial cross-talk. We thus expected that in the presence of plant extracts these AHL molecules might be blocked. Consequently, the biosensor bacterium cannot produce violacein pigment when they grow in the vicinity of Aeromonas and Pseudomonas on a plate containing herbal extracts, whereas without the extracts, the purple pigment will be clearly detectable.

We therefore first determined the MIC and sub-MIC values of the herbal extracts for these bacteria. A total of 200 μL of an overnight culture of CV026 was diluted to OD₆₀₀nm = 1 and then spread uniformly on the LB agar. Wells were punched in the centres of plates. Then 90 μL of each P. aeruginosa and A. veronii suspension (OD₆₀₀nm = 1) separately plus a sub-MIC of herbal extracts (10 μL) were added to each well. In parallel, in control plates, P. aeruginosa and A. veronii were not formerly mixed with any herbal extract. The plates were incubated for 48 hours at 28°C, and we evaluated the diameter of the signal diffusion zone during bacterial growth in the presence of herbal extracts by measuring the diameter of the violacein production zone by C. violaceum CV026 on LB agar.

Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed using ANOVA to compare the differences between tests and controls using SPSS Statistics 21 software (IBM, Armonk, NY, USA).

Results

Antibacterial susceptibility screening and MICs

The antimicrobial effects of Syzygium aromaticum, Dionysia revoluta Boiss. and Eucalyptus camaldulensis extract on bacterial QS are listed in Table 2. According to the results, all of the plant

| Characteristic | Gram-negative bacteria | Gram-positive bacteria |
|----------------|------------------------|-----------------------|
|                | Klebsiella pneumoniae  | Staphylococcus aureus  | Acinetobacter baumannii |
| Syzygium aromaticum | 15.0 ± 0.7             | 14.0 ± 0.8             | 15.0 ± 0.1             |
| Dionysia revoluta Boiss. | 15.0 ± 0.2             | 14.0 ± 0.8             | 15.0 ± 0.1             |
| Eucalyptus camaldulensis | 15.0 ± 0.3             | 14.0 ± 0.8             | 15.0 ± 0.1             |
| Tetracycline (30 μg)   | 22.0 ± 0.2             | 21.0 ± 0.3             | 22.0 ± 0.2             |

Plant extract genus and species abbreviations are as follows: EC, Syzygium aromaticum; SA, Dionysia revoluta Boiss. Data are shown as mean ± SD (mm) inhibition zone.

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extracts from 50 to 0.39 mg/mL had antibacterial effects, as shown through inhibition of bacterial growth compared to negative (DMSO) and positive (tetracycline) controls; these results were not significantly different (p < 0.05). The MBC, MIC and sub-MIC of these herbal extracts against A. veronii bv. Sobria BC88, P. aeruginosa and C. violaceum are shown in Table 3.

Inhibition of violacein production in C. violaceum CV026

According to the obtained results from 96-well microtitre, inhibition of violacein production was observed (OD<sub>600nm</sub>) after treatment with increasing sub-MICs of herbal extracts compared to control (Fig. 2). Interestingly, the growth of C. violaceum CV026 in the presence of plant extracts was not inhibited in the cell counts (OD<sub>600nm</sub> = 1) and showed no significant difference compared to control. Violacein inhibition was then compared to the control wells. The analysed data showed that Eucalyptus camaldulensis Dehnh. at 0.78 and 0.39 mg/mL, Syzygium aromaticum at 0.39 and 0.195 mg/mL and Dionysia revoluta Boiss. at 3.1 and 1.56 mg/mL concentration have a maximum violacein inhibition (>50%) at sub-MICs (Fig. 3). However, sublethal minimal QS inhibitory concentration through weak pigment inhibition was observed by Dionysia revoluta Boiss. at 0.39 mg/mL, Eucalyptus camaldulensis Dehnh. at 0.097 mg/mL and Syzygium aromaticum at 0.048 mg/mL, and no anti-QS activity was found at lower concentrations of plant extracts.

CV026 agar assay

The sub-MIC of each herbal extract, with maximum potential inhibition of violacein in 96-well microtitre results, showed an anti-QS effect on LB agar plates. This QS-inhibitory effect was observed for Eucalyptus camaldulensis Dehnh. at 0.78 mg/mL, Syzygium aromaticum at 0.39 mg/mL and Dionysia revoluta Boiss. at 3.1 mg/mL with the formation of a visible halo zone (growth without pigmentation) around the wells compared to the DMSO control (Fig. 4).

Inhibition of QS signal molecules in A. veronii and P. aeruginosa

After evaluation of QS inhibition in a biosensor strain, we studied the anti-QS effect of herbal extracts on A. veronii bv. Sobria strain BC88 and P. aeruginosa isolated from a hospitalized patient with cystic fibrosis. Results indicated that A. veronii and P. aeruginosa populations produced and diffused QS signal molecules during growth on LB agar, and C. violaceum CV026 could produce violacein pigments in response to these exogenously provided signal molecules when they were cultured next to each other. Meanwhile, in the presence of sub-MICs of herbal extracts, A. veronii and P. aeruginosa populations were constant (OD<sub>600nm</sub> = 1) but signal diffusion was decreased in LB agar. These results were demonstrated by a decrease in the zone of violacein pigment productions in C. violaceum (Figs. 5 and 6). The results confirm the anti-QS activity of the herbal extracts we assessed.

Discussion

QS, or bacterial talking, controls vital functions in both Gram-negative and-positive bacteria. As a result of the clinical, environmental and industrial applications of QS inhibitors, the search for potential compounds with anti-QS activity has recently intensified [36]. Overall, the inhibition of QS pathways with natural and synthetic compounds is undoubtedly a promising path to combating multidrug-resistant bacteria, which makes pathogens more susceptible to host immune responses and antibiotics. Over the past few decades, it has become increasingly clear that herbal medicines are a promising and abundant source of antibacterial and anti-QS compounds. In Middle Eastern countries, especially Iran, medicinal plants are used extensively to treat many diseases [37,38]. Even though Syzygium aromaticum, Dionysia revoluta Boiss. and Eucalyptus camaldulensis Dehnh. have been used for a long time in Iran and elsewhere around the world for a wide range of medical treatment, and even though evidence has demonstrated their antimicrobial activity and their ability to inhibit bacteria growth, the nature of their anti-QS properties remains unknown [18–23,37,38].

In the present study, we investigated the anti-QS activity of several medicinal plants. In vitro assays demonstrated the potential of Syzygium aromaticum, Dionysia revoluta Boiss. and

| TABLE 3. Characteristics of plant extract MICs in vitro |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Plant species                  | Chromobacterium violaceum CV026 | Pseudomonas aeruginosa          | Aeromonas veronii bv. Sobria BC88 |
|                                | MBC    | MIC    | Sub-MIC | MBC    | MIC    | Sub-MIC | MBC    | MIC    | Sub-MIC |
| Dionysia revoluta Boiss.       | 6.25   | 1.56   | 0.78    | 0.048  | 0.97   | 0.048   |
| Eucalyptus camaldulensis Dehnh.| 6.25   | 1.56   | 0.78    | 0.097  | 0.195  | 0.048   |
| Syzygium aromaticum            | 12.5   | 0.78   | 0.39    | 0.048  | 0.97   | 0.048   |

MBC, minimal bactericidal concentration (mg/mL); MIC, minimum inhibitory concentration (mg/mL); sub-MIC, sub–minimum inhibitory concentration used for anti–quorum-sensing assay (mg/mL).
Eucalyptus camaldulensis Dehnh. in inhibiting the production of QS-regulated virulence factors (violacein) in C. violaceum CV026. Furthermore, other studies in Iran have reported the anti-QS effects of some herbal extracts, such as Lepidium draba, Anethum graveolens, Raphanus sativus, Artemisia dracunculus and Althea officinalis, which reportedly inhibit QS in C. violaceum CV026 [40,41]. Hence, Mohabi et al. [42] showed the activity of Quercus infectoria gall extract on virulence factor production and inhibition of QS in Pseudomonas aeruginosa. Moreover, in other countries, studies have shown that the anti-QS and in vitro antibiofilm potentials of A. graveolens against uropathogenic Serratia marcescens or pompia and grapefruit essential oils potently inhibited biofilm formation, which could be used to control common polymicrobial infections [43,44]. Because QS disruption is an effective strategy to control infections caused by different bacteria that are pathogenic to plants, animals and humans, it can provide new opportunities in a wide range of applications, including therapeutic applications for both humans.
and animals. For example, today, anti-QS drugs, such as anti-QS–based antiseptic ointments, mouthwash for oral infections and drops for ear infections, have emerged. Similarly, functional foods such as plant products are rich in compounds with anti-QS activity for controlling infections in immunocompromised patients [36].

The present study assessed the anti-QS properties of Syzygium aromaticum, Dionysia revoluta Boiss. and Eucalyptus camaldulensis Dehnh. against C. violaceum CV026, A. veronii bv. Sobria BC88 and P. aeruginosa. Further studies are necessary to identify the major compounds responsible for the anti-QS activity present in the tested plants. We hope that the

FIG. 4. Results from quorum-sensing (QS) inhibition assay on Luria-Bertani agar plates. Anti-QS effect of Syzygium aromaticum (0.39 mg/mL), Dionysia revoluta Boiss. (3.1 mg/mL) and Eucalyptus camaldulensis Dehnh. (0.78 mg/mL) shown without pigmented colony. Dimethyl sulfoxide (DMSO) served as negative control and had no anti-QS effect.

FIG. 5. Results from anti–quorum-sensing (QS) activity through signal inhibition in Aeromonas veronii bv. Sobria BC88. In control well, exogenous QS signals were produced and diffused through A. veronii; these molecules were tracked with purple pigments produced by Chromobacterium violaceum CV026 in Luria-Bertani agar. At minimal bactericidal concentration, A. veronii was killed and could not diffuse signals, but in presence of sub–minimum inhibitory concentration (MIC) of herbal extracts, bacterial populations were fixed (OD600nm = 1), and QS signal diffusion zone was decreased compared to control (mean ± SD, p < 0.05).
present study can move research from the theoretical to the experimental.

Conclusions

Some natural plant extracts, especially Syzygium aromaticum, Dionysia revoluta Boiss. and Eucalyptus camaldulensis Dehnh., can be useful in the treatment of some kinds of infections during chronic disease by preventing and depleting the signaling communication between members of the bacterial community and by controlling the bacterial population, thus permitting the immune system to eradicate the infection.

Conflict of interest

None declared.

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