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Original Article

Anti-bacterial and Anti-Quorum Sensing Properties of *Dionysia Revolute Boiss* against Secondary Bacterial Infections of COVID-19 Patients; An *in-vitro* Study

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Abstract

Background and Aim: Today, the use of traditional plant compounds to kill or interfere with their quorum sensing (QS) mechanisms is considered as an alternative approach to control secondary bacterial infections during or after a viral infection. In this study, anti-bacterial and anti-quorum sensing effect of *Dionysia revolute Boiss* against five secondary bacterial infections of COVID-19 patients were evaluated.

Materials and Methods: Extraction of the plant compounds was carried out using *n*-hexane, methanol, and 96% ethanol mixed solvent. Bacterial samples were collected from respiratory tract fluids among COVID-19 patients and recognized with API kits. Antibacterial activity of the herbal extract was assessed by disc diffusion method as proposed by the Clinical Laboratory Standards Institute (CLSL, 2015). Hence, anti-QS activities of this herbal extract at the sub-minimum inhibitory concentration (MIC) were assessed by violacein quantification assay in *Chromobacterium violaceum* CV026 biosensor strains *in vitro*.

Results: As it has been indicated in the Results section, a plant extract from 50 to 0.39 mg/ml exposed their antibacterial impacts via hindering the bacterial growth in comparison with controls and exhibited anti-QS activities via decreasing the violacein formation in *C. violaceum* CV026 biosensor strain at sub-MIC concentrations (3.1 to 0.39 mg/ml) *in vitro*.

Conclusion: Our study showed that the antimicrobial activities of *Dionysia revolute* Boiss could be due to their anti-QS properties. Therefore, this medicinal plant either as a stand-alone treatment or in combination with antibiotics could be used as an efficient choice for curing secondary bacterial infections.

Keywords: *Dionysia revolute*, Anti-bacterial, Anti-quorum sensing; Secondary bacterial infections; SARS COVID-19

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Introduction

Hospitalized patients are exposed to secondary bacterial infections, which result in high mortality rates, during or after initial infection with an infective pathogen, often a virus. One of the recommended methods to control secondary bacterial infections during or after a viral infection is the use of traditional medicinal plant compounds to kill or interfere with their quorum sensing (QS) mechanisms which are vital for biological behavior to provoke an immune response in host defense against bacterial infections. For instance, after the appearance of severe acute respiratory syndrome (SARS), and coronavirus (COVID) as a global pandemic in 2019, controlling the secondary bacterial infections as a nosocomial infection in SARS COVID-19 patients, acquired from intensive care units in hospitals, became very challenging because the unrestrained consumption of antibiotics in human medicine therapy and formation of the bacterial biofilms are considered the major factors that significantly reduce the sensitivity of nosocomial bacterial pathogens to antibacterial agents (1-8). Moreover, an optional procedure to deal with drug resistant in secondary bacterial infections is the use of natural and traditional antimicrobial agents such as medicinal plant compounds, fruit and vegetables. They are used to combat with bacterial infections by killing them or producing distinct antimicrobial compounds, such as phenolic, terpenoids, flavanones, and quinones due to similarity in their chemical structure to those of QS signals (AHL) and the ability to degrade signal receptors or interfere with the QS-regulated gene expression in the invading organism (4, 9-15). In Middle East regions like Iran, antimicrobial activities of herbal medicines against certain bacterial species have been reported, particularly from the extracts and fractions of golden stone bride with the scientific name *Dionysia revolute* Bioss & the plant family Primulaceae. Golden stone bride is the name of a species of the stone bride genus. There are about 27 species of this genus in Iran, which are mostly exclusive to Iran and grow in rocky deserts and rock crevices. The main chemical components of this herbal extract are 2-Acetophenone, benzaldehyde, 2- acetyl phenol, β-farnesyl alcohol, eugenol, rosifoliol, γ-eudesmol, and o-hydroxychalcone (16-19, 44). Furthermore, some researches have investigated and reported antifungal, anti-inflammatory, anti-cancer and antioxidant impacts of this herbal medicine (18, 20-22). Hence, the present study was conducted to examine the antibacterial effects of this herbal extract against five nosocomial gram negative and positive bacteria that were isolated from respiratory tract infections in hospitalized patients with SARS COVID-19. Furthermore, to validate the antimicrobial claim of this traditional medicinal plant and to find out whether the antimicrobial effects of this medicinal plant are associated with interference with bacterial QS systems, the effective sub minimum inhibitory concentrations (MIC) of this herbal extract on the QS system of *Chromobacterium violaceum* CV026 biosensor strain (mini-T5-mutant of the wild type strain employed widely for QS studies) were evaluated by violacein quantification assay *in vitro*.

Materials and Methods

Collection of Plant Materials and Extract Preparation: Plant materials were collected from Fars province, Iran. Confirmation of a voucher specimen and scientific identity of the plant compound were confirmed at the Shiraz University of Medical Sciences Herbarium Department. Deposition of the voucher specimen was carried out in the Herbarium of Shiraz Faculty of Pharmacy, Shiraz, Iran (Table1). Initially, we washed the plant sample twice with distilled water and then dried it in the dark place at 25°C for 72 hours. Subsequently, we powdered the dried plant sample and submerged it 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 yield of the extract was calculated using the following formula:

Yield (%) = [dry crude extract / dry initial plant material before extraction] × 100

We filtered and concentrated the plant extract with a rotary evaporator and solved it in adequate

concentrations of dimethyl sulfoxide ((DMSO), 50 to 0.048 mg/ml) until further investigation.

Bacterial Isolation and Culture Conditions: For the antibacterial assays, five bacterial strains (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus and Serratia marcescens*) were provided from respiratory tract infections in hospitalized COVID-19 patients in the clinical laboratory of Peymaniyeh Hospital, Jahrom, Iran. Respiratory tract fluids were cultured on blood agar and EMB (Merck) and primary isolation was performed after 24 hours incubation at 37°C. Bacterial samples were recognized by colony morphology, biochemical tests and API kits (bio Merieux's API kit) for Gram-negative (API 20E '18-24 h; identification of Enterobacteriaceae and other non-fastidious Gram-negative bacteria) and Gram-positive bacteria (API 20 Strep "4-24h; identification of streptococci & staphylococci). Hence, *Chromobacterium violaceum* CV026 (mini-Tn5 mutant of wild type strain, National Collection of Type Culture 13278, -80°C stock in Bacteriology and Virology Department of Shiraz University of Medical Sciences) was used for QS inhibition assay. The C6-AHL was used in the present research as a signal molecule purchased from Sigma Aldrich. *C. violaceum* CV026 cultured in Luria-Bertani medium (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract) was supplemented with kanamycin (30 µg/mL) and chloramphenicol (30 µg/mL) with shaking at 220 rpm, 28°C.

Antibacterial Properties: In the present study, the disc diffusion method was used based on the guidelines proposed by Laboratory Standards Institute (CLSL, 2015) and Murray *et al*., 2016 in order to investigate the antibacterial activity of the herbal extract on bacterial strains (37, 41). The herbal extract was dissolved in DMSO (50-0.39mg/ml), and Mueller Hinton Agar Plates (Merck, Germany) were inoculated uniformly with 1 ml of each bacterial suspensions (10^8 CFU/ml). Hence, sterile paper discs (6 mm) were loaded by herbal extracts dilutions (50 µl) and set on the bacterial cultured plate. Subsequently, they were incubated at 37°C for 48 hrs. Tetracycline (30 µg) and DMSO were used as positive and negative controls (37). Antibacterial activity of the herbal extract was examined via measuring the diameter of inhibition zone in mm.

Minimal Inhibitory Concentrations (MICs): Prior to anti-QS assay, the MBC (minimal bactericidal concentrations), MIC and sub-MIC values of the herbal extract were measured using serial Macro dilution assay in control and test tubes according to the guidelines proposed by Laboratory Standards Institute (CLSL, 2015) (41). Initially, *C. violaceum* CV026 was cultivated overnight at 37°C in LB broth. The herbal extract was initially tested at 25 mg/ml and serially diluted in two-fold (control and test tube), to 0.048 mg/ml. Every test tube contained 0.5 ml of each concentration and was inoculated with identical volumes of the microbial suspension (10^6 CFU/ml). However, the control tubes merely contained herbal extracts concentration and broth media without bacterial inoculum to be used as a blank. This was carried out to do away with plant extract and broth media turbidity in order to follow bacterial growth in the test tube in spectrophotometric assay at 600 nm. We used an extract-free tube with microbial suspension as a positive control. The incubation of all of the tubes was carried out at 37°C for 24 h. The tubes that were not characterized by bacterial growth exhibited bactericidal impacts, and the lowest concentration of the plant extract that could at least inhibit 50% of microorganism's growth was regarded as MIC by the use of the following equation (23):

$$\text{Inhibition \%} = [(\text{OD C} - \text{OD T}) / \text{OD C}] \times 100$$

OD C is OD600 nm to track bacterial growth in the positive control tube, and OD T is OD600 nm to make an estimate of bacterial growth in the test tubes. To make certain about the presence or absence of bacterial growth in the test tubes, a standard loop of the suspensions in every tube was inoculated on 3mm MHA and incubated overnight at 37ºC.

Violacein Quantification Assay: To examine the anti-quorum sensing capability of sub-MIC concentration of the herbal extract in a microtube, assays were carried out to investigate the inhibition of violacein production in *C. violaceum* CV026**.** An overnight culture of C. *violaceum* CV026 diluted 1:50 in 4 mL of fresh LB broth, supplemented with (0.25 µg/ml) N-acyl homoserine lactone (C6-AHL) and different sub-MIC concentrations of herbal extracts. In this assay, the control containing *C. violaceum* CV026

and LB broth was used to control bacterial population without pigment production (negative control, OD_{600} =1) and C. *violaceum* CV026, and AHL signal without herbal extracts to examine violacein production in the constant bacterial population (positive control). Microtubes were incubated for 24 hours at 28°C in a shaking incubator and the absorbance of each Microtube was read in 585 and 600nm for violacein production and bacterial population growth, respectively by a Polar Star Omega Microplate reader (BMG LAB TECH, Germany). The assay was performed again in triplicate and the percentage of violacein pigment inhibition in comparison with the control microtube was calculated by the use of the following equation [24, 25]: inhibition (%) = [(OD 585 C – OD585 T) / OD585 C] × 100.

In this formula, OD C is OD585 for violacein production in the control microtube (without herbal extracts) and OD T is OD585 for violacein production in the presence of herbal extracts by *C. violaceum* CV026. To make sure that the inhibition of violacein production was not caused by antibacterial activity, the impact of herbal extracts on the growth of *C. violaceum* CV026 was further investigated through the comparison of bacterial population in all of the microtubes with the controls (without herbal extracts) in OD600=1. The constancy of bacterial population in the all of the microtubes indicated that the inhibition of violacein production was caused by anti-quorum sensing effects.

***C. Violaceum* CV026 Assay:** To carry out *C. violaceum* CV026 assay, an overnight culture of CV026 was diluted to (OD600 = 1), and 200 µl of it was spread circularly at the middle of LB agar plates that was supplemented with C6-AHL (0.25 µg/ml). Microtubes were punched in the center of culture plate following the incubation that continued for 30 min. Sub-MIC concentrations of plant extracts (50 µL) with maximum anti QS impacts were acquired from the previous experiment. Subsequently, they were inoculated in every microtube. DMSO was used as negative control. Incubation of the plates was carried out at 28°C for 48 hours. A white circle of bacterial growth, surrounded by a purple halo around the microtube (filled with the plant extract), meant that the extracts exhibited anti-QS via inhibiting the violacein production around the microtube. Upon the extreme dilution of the diffused molecules of plant extract, we saw the purple color once more, which could be observed as an outer purple circle (26).

Statistical Analysis: All the experiments were conducted in triplicates and statistical analyses were performed by the use of ANOVA to compare the distinctions between tests and controls using SPSS statistics 21. The research project was approved by the ethical committee of the Shiraz University of Medical Sciences (ethical approval No. IR. SUMS. - REC.1397.083)

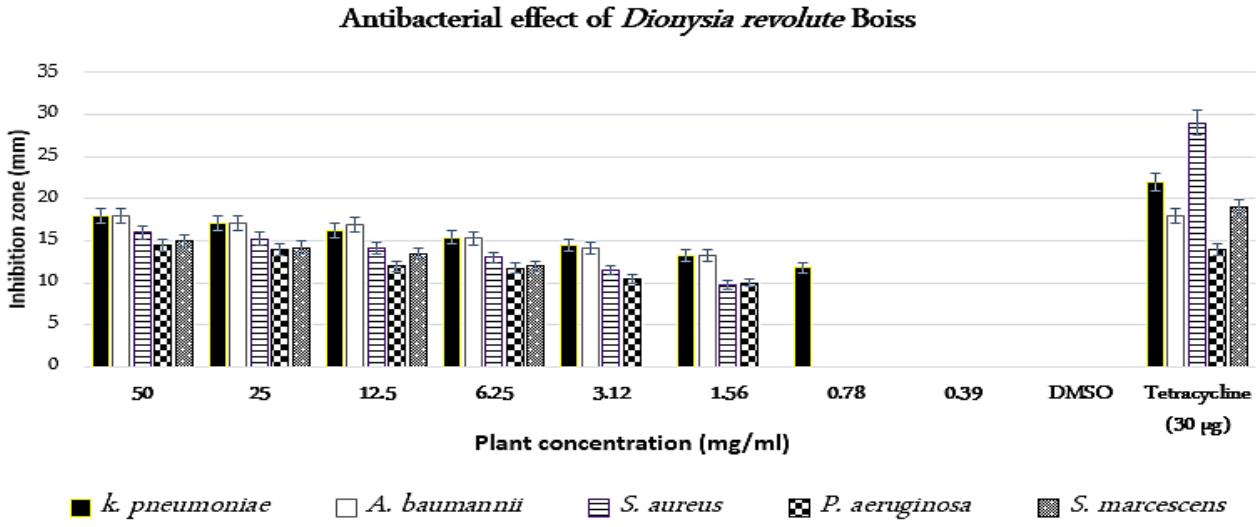

Figure 1. Antibacterial effect of plant extracts on gram negative and gram-positive bacteria. The The experiments were performed in triplicates and the results were expressed as mean ±SD.

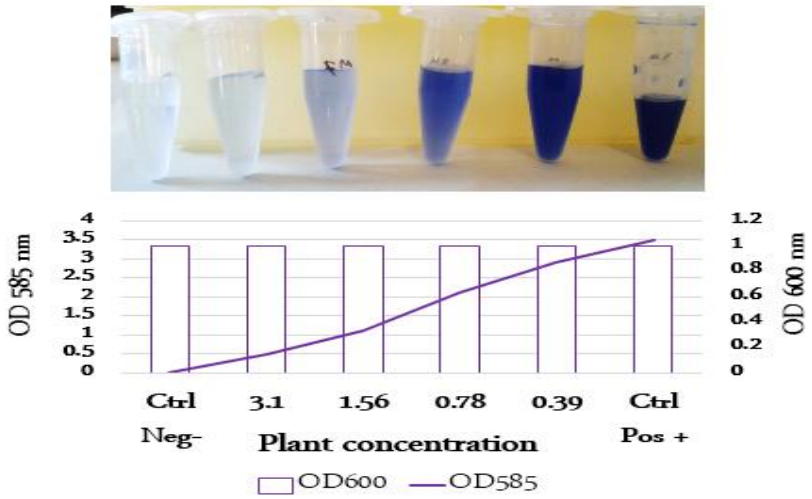

Figure 2. The result from Violacein quantification assay in a Microtube. Bacterial population was stable in the presence of sub-MIC concentration of herbal extracts (OD600nm = 1); hence, the inhibition of Violacein production was observed following treatment by increasing sub-MIC concentration of herbal extract in comparison with controls (OD585nm). The experiments were performed in triplicates and the results expressed as mean ±SD.

Results and Discussion

Antibacterial Susceptibility Screening and MICs: Before investigating the impacts of *Dionysia revolute* Boiss extract on bacterial QS, their antimicrobial activities were listed in Fig 1. As the results indicate, plant extract from 50 to 0.39 mg/ml exhibited their antibacterial effects via the inhibition of bacterial growth in comparison with negative (DMSO) and positive (Tetracycline) controls, and these findings were not remarkably distinct ($P < 0.05$). Moreover, The MBC (25mg/ml), MIC (6.25mg/ml) and sub-MIC (3.1-0.39mg/ml) concentration of this herbal extracts against *C. violaceum* were evaluated. Finally, the interference of herbal extracts in sub-MIC

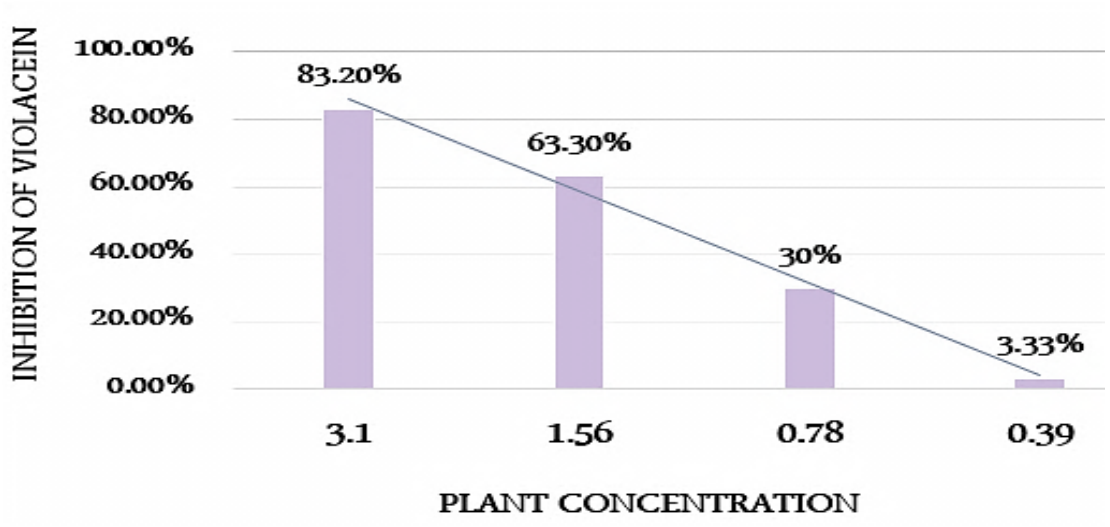

Figure 3. The results of the calculation of violacein inhibition percentage in the microtube using the following equation: Inhibition (%) = [(OD 585 C – OD585 T) / OD585 C] × 100. OD C is OD585 for violacein production in the control tube (without herbal extracts) and OD T is OD585 for violacein production in the presence of herbal extracts. *D. revolute* at (3.1, 1.56 mg/ml), showed maximum reduced violacein production in *C. violaceum* CV026.

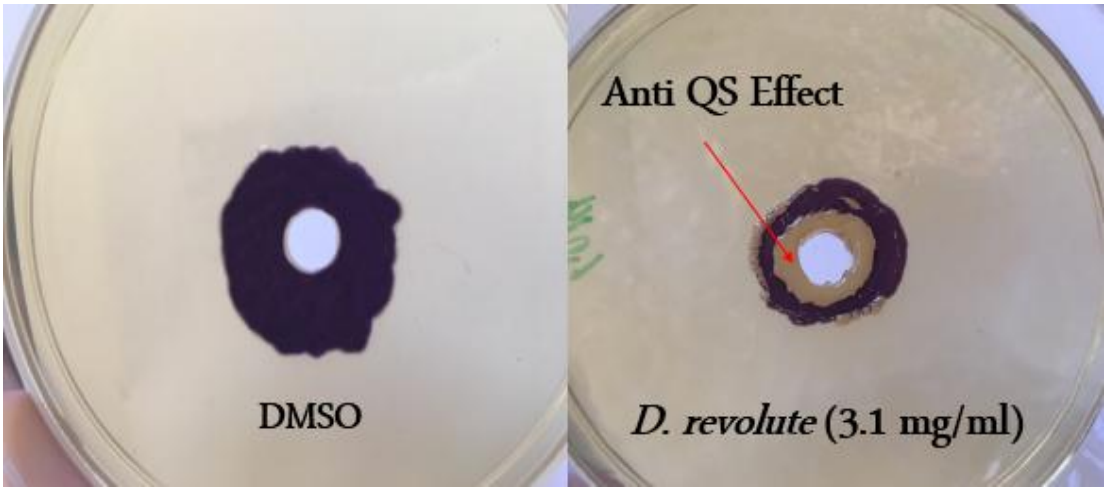

Figure 4. The results of QS inhibition assay on LB agar plates. Anti QS effect of *Dionysia revolute* Boiss (3.1 mg/ml) showed without pigmented colony. DMSO served as the negative control and without anti QS effect.

concentrations with bacterial QS was examined in acyl homoserine lactone (C6-AHL)-based *in-vitro* QS competition assay using *C. violaceum* CV026 as the test strain.

Inhibition of Violacein Production in *C. Violaceum* CV026: After determining the herbal extract' MICs on *C. violaceum* CV026, anti-quorum sensing effects of the herbal extract in sub-MIC concentrations were conducted in order to determine the inhibition of violacein production in *C. violaceum* CV026. According to the results, inhibition of violacein production occurred (OD585nm) after treatment with increasing sub-MIC concentration of herbal extracts compared to the controls (Fig 2). Interestingly, the inhibition of the growth of *C. violaceum* CV026 in the presence of plant extracts did

Table 1: Information about the plant compound extracted in this study.

*Voucher	1093
Plant Species	*Dionysia revolute Boiss.*
Plant Family	Primulaceae
Common Name	Golden Stone Bride
Tested Part	Whole plant
Weigh (g)	88
Solvents (mixed)	*n*-hexane, methanol, 96%ethanol
Yield (%)	3.8%
Extract colors	brown
Clinical Usage/Reference	Antimicrobial, Anti-inflammatory, Anti cancer/[18,19]

*A voucher specimen was deposited in the Herbarium of Shiraz Faculty of Pharmacy, Shiraz, Iran

not occur as the cell counts (OD600nm = 1), and no remarkable distinction in comparison with the controls was observed. Subsequently, we compared the violacein inhibition with the control microtube, and the analyzed data revealed that *Dionysia revolute* Boiss (3.1 & 1.56 mg/ml) could have a maximum violacein inhibition (>50%) at the sub-MIC concentration (Fig 3). Nevertheless, a weak pigment inhibition was seen by Dionysia revolute Boiss at 0.78, 0.39 mg/ml, and no activity was found at lower concentrations of plant extracts.

CV026 Agar Assay: Sub-MIC concentration of every herbal extract (1/2 MIC), with the maximum potential inhibition of violacein in a 96-microplate results indicated anti QS impacts on the LB agar plates. This QS-inhibitory impact was seen for *Dionysia revolute* Boiss at 3.1mg/ml with the formation of a visible halo zone (growth without pigmentation) around the wells in comparison with the controls (DMSO) (Fig 4).

Although numerous research and clinical studies have meticulously investigated primary pathogens, particularly in prevalence of coronavirus as a global pandemic, research on secondary bacterial infection(s) has been neglected. Recently, the use of traditional medicinal plants with antimicrobial properties expanded to include controlling bacterial infections because they have abundant bactericidal and anti-infective compounds to inhibit and suppress the bacterial pathogens (42, 43). The use of these medicinal plants can be helpful to treat secondary bacterial infections, by inhibiting or reducing the pathogenicity of bacteria before killing the agents. For instance, secondary bacterial infections in patients with pulmonary coronavirus infection which are defined as nosocomial infections with high mortality rates could develop after 72 h in hospitals, particularly in intensive care units (ICU). The prevalence of these nosocomial infections during a primary COVID-19 disease may be due to the damage or deletion of mucosal layer in respiratory tracts and unknown variations in host immune responses. Hence, the use of broad-spectrum antibiotics to cure secondary bacterial infections is a principal strategy in hospitals which can develop antimicrobial resistant (27,28). Medicinal plants with antibacterial effects can be promising choices for treating nosocomial infections, particularly in secondary bacterial infections with antibiotics resistance. On the other hands, the inhibition of QS pathways which control the central role both in Gram-negative and positive bacteria with natural and synthetic compounds is obviously helpful in fighting multidrug-resistant bacteria, which make pathogens more vulnerable to host immune reactions and antibiotics. During the past few decades, it has been revealed that herbal medicines are plentiful sources of antibacterial and anti-QS compounds. In the Middle-Eastern countries, particularly in Iran, medicinal plants are widely used in the treatment of many diseases (29-31). Even though medicinal plants, particularly *Dionysia revolute* Boiss, have been traditionally used in Iran for treating various diseases, and the evidence has indicated their antimicrobial activities, the anti-quorum sensing properties of this herbal extract have still remained unknown (18-22, 30-33). In this research, we examined the anti-bacterial and anti-QS activities of this medicinal plant. We showed the ability of *Dionysia revolute* Boiss to hinder the production of QS-regulated virulence factors (violacein) in *C. violaceum* CV026. Hence, our study revealed that the antimicrobial activity of *Dionysia revolute* Boiss could be due to its anti-QS properties. Therefore, this medicinal plant either as a stand-alone treatment or in combination with antibiotics might present an efficient remedy in the treatment of secondary bacterial infections in Sars-COVID19 patients. In line with our study, several other studies conducted in Iran have indicated the anti-QS impact of some herbal extracts, such as *Lepidium draba* and *Anethum graveolens*, *Raphanus sativus*, *Artemisia dracunculus*, *Althea officinalis*, which have been reported to inhibit quorum sensing in *C. violaceum* CV026 (34, 35). Therefore, Mohabi *et al*. indicated the activity of *Quercus infectoria* galls extract on the production of virulence factor and inhibition of quorum sensing (QS) in *Pseudomonas aeruginosa* (36). Furthermore, the anti-QS and *in-vitro* anti-biofilm potential of *A. graveolens* against uropathogenic *Serratia marcescens* or pompia have been indicated in some studies conducted in other countries. Moreover, it has been shown that grapefruit essential oils can efficiently inhibit biofilm formation, which could be used to control common polymicrobial

infections (37, 38). To develop healthcare emergency from Sars-COVID19 and antibiotic-resistance of secondary infections, medical interventions using experiential therapy through medicinal plants with anti-bacterial and anti-QS effects might be our most promising options that can provide new alternatives with several uses, such as therapeutic uses (27). This study attempted to explore the anti-bacterial property of *Dionysia revolute* Boiss against some secondary bacterial infections isolated from patients with SARS COVID-19 and anti-QS in *C. violaceum* CV026 biosensor strain. Further research is required to identify the major constituent responsible for anti-QS activity found in the tested plants. Hopefully, this research is able to move from theoretical researchers to experimental ones.

Conclusion

In summary, our indicated that the antimicrobial properties of *Dionysia revolute* Boiss can be due to its anti-QS activities. It was shown that some natural plants extract, especially the *Dionysia revolute* Boiss, is one of the most efficient alternatives for curing secondary bacterial infections in chronic diseases, particularly in patients with SARS COVID-19 with treatment alone or in combination with broad-spectrum antibiotics. It can make pathogens more vulnerable to host immune reactions and antibiotics. Moreover, these results can be valuable for the prevention and depletion of signaling communication between bacterial communities and regulating the bacterial population to leave it to the immune system to cure the infection.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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