

# Antibacterial, anti-swarming and anti-biofilm formation activities of *Chamaemelum nobile* against *Pseudomonas aeruginosa*

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## ABSTRACT

**Introduction:** Chamomile (*Chamaemelum nobile*) is widely used throughout the world, and has anti-inflammatory, deodorant, bacteriostatic, antimicrobial, carminative, sedative, antiseptic, anti-catarrhal, and spasmolytic properties. Because of the increasing incidence of drug-resistant bacteria, the development of natural antibacterial sources such as medical herbs for the treatment of infectious diseases is necessary. Extracts from different plant parts such as the leaves, flowers, fruit, and bark of *Combretum albiflorum*, *Laurus nobilis*, and *Sonchus oleraceus* were found to possess anti-quorum sensing (QS) activities. In this study, we evaluated the effect of *C. nobile* against *Pseudomonas aeruginosa* biofilm formation. **Methods:** The *P. aeruginosa* samples were isolated from patients with different types of infection, including wound infection, septicemia, and urinary tract infection. The flowers of *C. nobile* were dried and the extract was removed using a rotary device and then dissolved in dimethyl sulfoxide at pH 7.4. The microdilution method was used to evaluate the minimum inhibitory concentration (MIC) of this extract on *P. aeruginosa*, and biofilm inhibition was assayed. **Results:** Eighty percent of the isolated samples (16/20) could form a biofilm, and most of these were isolated from wound infections. The biofilm inhibitory concentration of the *C. nobile* extract was 6.25-25mg/ml, whereas the MIC was 12.5-50mg/ml. **Conclusions:** The anti-QS property of *C. nobile* may play an important role in its antibacterial activity, thus offering an additional strategy in the fight against bacterial infections. However, molecular investigation is required to explore the exact mechanisms of the antibacterial action and functions of this phytochemical.

**Keywords:** Anti-quorum sensing (QS). Antibacterial. *Chamaemelum nobile*. Biofilm. *Pseudomonas aeruginosa*.

## INTRODUCTION

The 21<sup>st</sup> century has seen a reversion from chemical drugs to increased interest in the utilization of natural products for medicinal purposes, particularly plants. Accordingly, there have been many developments with respect to investigations of medical herbs, and this field is expected to expand in the future. The use of dried herb extracts (total extracts) instead of purified herb materials can increase the efficiency of an herb's medical effects with less side effects, because of the diluted concentration of the plant material. Such extracts come in various forms such

as tablets, capsules, ointments, syrup, and others, which are suitable for contemporary lifestyle. Because of the increasing demand for medical herbs for the treatment of various ailments and conditions, more research in this field is essential<sup>(1)(2)(3)</sup>.

Several plants with antibacterial effects are commonly used in medicine, such as chamomile (*Chamaemelum nobile*), which is a small aromatic plant that is a traditional medical herb with anti-inflammatory, antimicrobial, and antiseptic properties. It has long been used for the treatment of several conditions such as malaria, peptic ulcers, wound healing, prevention of gangrene, and others<sup>(4)</sup>.

Many host-associated bacteria use chemical signals to monitor their own population density and to control the expression of specific genes in response to population density. This type of gene regulation is termed quorum sensing (QS)<sup>(5)</sup>, which is a general phenomenon identified in many gram-negative and gram-positive bacteria<sup>(6)(7)</sup>. Many gram-negative bacteria employ N-acyl homoserine lactones (AHLs) as QS signaling compounds, which vary among bacteria with respect to length and the specific substitutions of their acyl side chains. The signaling molecule

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is synthesized by a LuxI-type synthase and binds to a cognate LuxR-type transcriptional activator protein to regulate the expression of target genes. At low cell density, the signaling compound is synthesized at a low basal level and is thought to diffuse into the surrounding medium where it becomes diluted. However, during growth, the AHL concentration in the medium rises to a critical threshold. At this concentration, AHL becomes activated by binding to its cognate receptor, and consequently stimulates or represses the transcription of target genes<sup>(8)</sup>. Such a QS process regulates important bacterial activities such as virulence gene expression and biofilm formation.

Biofilms are a highly structured, surface-attached community of cells enclosed in a self-produced polymeric matrix. In laboratory-based systems, *Pseudomonas aeruginosa* forms a biofilm (several hundred micrometers in thickness) with tower- and mushroom-shaped microcolonies intervened by water channels and void spaces<sup>(9)</sup>. *Pseudomonas aeruginosa*, a gram-negative opportunistic human pathogen, is responsible for persistent and often incurable infections in immunocompromised people or patients with cystic fibrosis<sup>(10)</sup>.

The most recent strategies aimed at discovering new inhibitors that are able to disrupt the different steps of the QS system have focused on targeting signal production, signal molecules, and signal receptors<sup>(11)</sup>. To overcome the biofilm formation problem of antibiotic-resistant bacteria, an anti-pathogenic approach has been recently considered as a viable alternative, and inhibition of QS is a particularly attractive target. Thus, researchers have focused on plant-derived antimicrobials as an alternative to the more ineffective antibiotics<sup>(12)</sup>.

Therefore, the development of natural antibacterial elements with other sources such as medical herbs is essential for novel treatments of infectious diseases<sup>(13)</sup>. Considering its reported curative properties, we decided to evaluate the effect of *C. nobile* against biofilm formation of *P. aeruginosa*.

## METHODS

### Samples

Samples were isolated from different infection sources, including wound infection, septicemia, and urinary tract infections, from 20 patients of Imam Khomini Hospital in Ilam City. We selected the strains with multidrug-resistant features (i.e., resistant to cloxacillin, cotrimoxazole, cephalosporin, carbenicillin, piperacillin, and ceftazidime). Isolates from patients were cultured in trypticase soy broth (TSB) and were stored in -70°C, and then freshly sub-cultured on brain heart infusion (BHI) agar prior to each assay.

### Herbal extraction

*Chamaemelum nobile* plants were collected from a wild-growing population at the full-flowering stage, from the mountains of the Ilam province of Iran. The flowers were dried out and the extracts were removed using a rotary device. The acquired suspension was then concentrated via a vacuum distillation process. To make the suspension, the extract was

dissolved in dimethyl sulfoxide at pH 7.4. The antibacterial properties of the extracts were initially determined using the broth microdilution method, and the dry weight of the extracts was calculated per milliliter.

### Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) values of plant extracts were determined by the broth microdilution method using 96-well microtiter plates. Serial two-fold dilutions of the plant extracts were prepared by vortexing the extracts in Millipore water. The inoculum of microorganisms was prepared in Mueller-Hinton Broth (Himedia, India), and the turbidity was adjusted to 0.5 McFarland and diluted to obtain a final turbidity in wells of approximately  $1 \times 10^6$  colony-forming units (CFU)/ml. Fifty micro liters of plant extract solution and 150µl of bacterial inoculum were placed into the wells of a microtiter plate and incubated at 37°C for 24h. The MIC is defined as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism. Then, the minimum bactericidal concentration (MBC) value was determined using standard methods. In brief, the MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by  $\geq 99.9\%$ <sup>(14)</sup>. *Pseudomonas aeruginosa* (PAO1) was used as a positive control.

### Data interpretation

Absorbance values lower than the control cells indicated a reduction in the rate of cell proliferation. Conversely, a higher absorbance ratio indicated an increase in cell proliferation. Rarely, an increase in proliferation or morphological changes may be offset by cell death. The percent inhibition was calculated with the following formula: % inhibition =  $1 - (\text{OD observed}/\text{OD control}) \times 100$ .

### Biofilm inhibition assay

One colony of each *P. aeruginosa* isolate was used for inoculation in 5ml of BHI broth. The culture was incubated for 18h at 37°C with aeration at 200rpm. Following incubation, the number of cells in each culture was adjusted to  $1.5 \times 10^8$  CFU/ml (0.5 McFarland), and 50µl of each bacterial suspension was transferred to eight wells of a 96-well microtiter plate. Eight replicates were used for each isolate in each biofilm assay. The biofilm-forming *Staphylococcus epidermidis* strain RP62A and PAO1, which are known to form fully established biofilms, were added to each plate as positive controls. Brain heart infusion broth was incorporated as a negative control. A 96-peg plate was then positioned in the wells of the microtiter plate, allowing the pegs to be submerged within the bacterial culture. The inoculated peg plate was transferred to a 96-well microtiter plate containing fresh BHI broth and incubated for 48h at 37°C on a rocking platform, to allow mature biofilms to establish. Each biofilm assay was repeated twice independently.

Biofilm biomass was quantified using a modification of a methodology described by Mowat et al<sup>(15)</sup>. Following incubation, the peg plate was removed from the microtiter plate, rinsed twice in phosphate-buffered saline to remove loosely attached

planktonic cells, and dried for 30 min at 37°C. Each replicate peg was stained with filtered 0.5% (w/v) crystal violet for 5 min. Excess crystal violet was removed by gently washing the peg plate twice with distilled water. Replicate pegs were detached from the plate using needle-tipped pliers and added to 1ml of 70% ethanol to leach the crystal violet from the stained biofilms. The absorbance at 570nm was measured using a microtiter plate reader. As the polystyrene pegs were suspended in the wells of the microtiter plate, any biomass that remained bound to the surface following the washing steps could be considered to be a genuine biofilm. The wells of the microtiter plate were not sampled for the presence of biofilm biomass, as this could have instead been a deposit of planktonic cells.

The capacity of each strain to form a biofilm was compared with the confluent biofilm control by analyzing the absorbance of the crystal violet stain obtained for each biofilm. This allowed for each isolate to be assigned a percentage value depending on the proportion of biofilm biomass it could establish after 48h in comparison with the control (assuming a rate of 100% for the control). Eight replicate pegs were included for each isolate in each biofilm assay and the assay was carried out three times. Isolates were also divided into three groups depending on whether they formed fully established biofilms with 75% of the biomass of the positive control, moderately adherent biofilms with 25-75% of the control biomass, or weak biofilms with 25% of the biomass of the positive control.

To evaluate the effect of *C. nobile* in inhibiting biofilm formation, 100µl BHI broth was added to a 96-peg plate, and 50µl of the extract was transferred to each of eight wells and diluted. A 50-µl aliquot of each bacterial suspension (0.5 McFarland) that formed a biofilm (see above) was transferred to eight wells of a 96-well microtiter plate and incubated for 24h at 37°C.

### Swarming assay

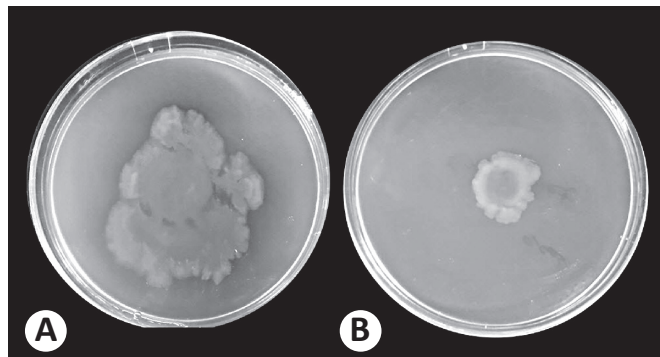
Fifty microliters of extract was mixed with 10ml of molten Mueller-Hinton agar medium and poured immediately over the surface of the plate as an overlay. The plate was point-inoculated with an overnight culture of PAO1 once the overlaid agar had solidified, and incubated at 37°C for 3 days. The extent of swarming was determined by measuring the area of the colony<sup>(16)</sup>. The experiment was performed in triplicate and repeated twice.

## RESULTS

A total of 20 samples were collected from different sources of infection in patients that had been referred to Imam Khomini Hospital of Ilam, 16 (80%) of the samples formed a biofilm. Of sixteen biofilm-forming *P. aeruginosa*, nine strains isolated from wound infections.

*Chamaemelum nobile* extract showed biofilm inhibitory activity in the tested concentration range of 1.6 to 100mg/ml. The effective concentrations against biofilm formation were 6.25-25mg/ml, whereas the MIC and MBC were 12.5-50mg/ml and 25mg/l, respectively.

Flagella motility-dependent swarming is also regulated by QS. Therefore, a reduction in swarming area compared with the control plate would suggest the presence of anti-QS compounds. *C. nobile*, which shows anti-swarming activity, selectively and distinctly reduced the swarming area of PAO1, suggesting that these extracts could inhibit the swarming motility of PAO1 (**Figure 1**).



**FIGURE 1 - Swarming inhibition of *Pseudomonas aeruginosa* PAO1 by *Chamaemelum nobile* extract. A) *Pseudomonas aeruginosa* PAO1 only (without extract). B) *Pseudomonas aeruginosa* PAO1 with *Chamaemelum nobile* extract. PAO1: standard strain of *Pseudomonas aeruginosa*.**

## DISCUSSION

Quorum sensing is a population density-dependent mechanism that is mediated through small signaling molecules called autoinducers by which bacteria regulate gene expression; in gram-negative bacteria, these autoinducers are AHLs. In some pathogenic bacteria, the QS system controls the expression of genes responsible for the production of virulence factors. Therefore, targeting QS has emerged as an alternative strategy for controlling bacterial virulence, and studies have identified both synthetic and natural compounds capable of disrupting QS, including some phytochemicals<sup>(17)</sup>.

The aim of this study was to evaluate the effect of chamomile extract on the biofilm formed by *P. aeruginosa*.

*Chamaemelum nobile* is widely used throughout the world, primarily as a sedative, anxiolytic, antispasmodic, and for the treatment of mild skin irritation and inflammation. Chamomile has anti-inflammatory, deodorant, bacteriostatic, antimicrobial, carminative, sedative, antiseptic, anti-catarhal, and spasmolytic properties<sup>(18)</sup>.

To the best of our knowledge, there are no published data about the anti-QS activity of *C. nobile* extract on *P. aeruginosa*. However, anti-streptococcal and antioxidant activities of essential oils of *C. nobile* have been reported<sup>(19)</sup>.

Recently, several potential quorum sensing inhibitory (QSI) have been discovered from various resources<sup>(20)</sup>. *Curcuma longa* produces curcumin, which inhibits the expression of virulence genes of *P. aeruginosa* PAO1<sup>(21)</sup>.



Furthermore, extracts from different plant parts such as the leaves, flowers, fruit, and bark of *Combretum albiflorum*, *Laurus nobilis* and *Sonchus oleraceus* were also found to possess anti-QS activities<sup>(22) (23)</sup>.

Vattem et al. investigated the anti-QS properties of several fruits, plants, and spices at a concentration of 0.5 mg/ml in the US in 2007. Their results indicated that among all of the extracts tested, only raspberry, blueberry, and grape extracts inhibited AHL activity-mediated violacein production by 60%, 42%, and 20%, respectively. Furthermore, kale, basil, and turmeric decreased PAO1 swarming by 41%, 35%, and 32%, respectively<sup>(24)</sup>.

In 2012, Paekiavathy et al.<sup>(25)</sup> investigated the anti-biofilm and anti-QS properties of cumin on gram-negative bacteria such as *P. aeruginosa* and *Chromobacterium violaceum*. They found that violacein production was inhibited by 2mg/ml of the cumin extract, and AHL and biofilm formation were also reduced<sup>(25)</sup>. Although we used a different extract, these results collectively indicate that plant extracts inhibit QS.

In 2011, Alipour et al.<sup>(26)</sup> investigated the inhibitory effects of an aqueous extract of the roots of American ginseng (NAGE) on QS, twitching, and biofilm formation of *P. aeruginosa* in Canada, and found a MIC and biofilm inhibitory concentration of 1.25-1.5% (w/v) (12.5-25mg/ml) and 5% (w/v) (50mg/ml), respectively. Therefore, the MIC concentration was similar to that observed in the present study, but the QS inhibitory concentration differed between NAGE and chamomile extracts<sup>(26)</sup>.

Furthermore, In 2011, Wu et al.<sup>(27)</sup> also investigated the inhibitory effects of NAGE on biofilm formation by *P. aeruginosa* and found that 5-20mg/ml of NAGE extract inhibited biofilm formation but not bacterial growth<sup>(27)</sup>. These results are concordant with the Alipour et al.<sup>(26)</sup> study with NAGE, but differ to our study in chamomile, suggesting that the difference is due to the specific extract tested. Moreover, it seems that the inhibitory effect of *C. nobile* extract on QS is greater and more efficient than that of NAGE. In our study, the MBC value was very close to the MIC value, which is considered to be a positive feature for any antimicrobial agent.

The formation of a biofilm from agents causing infections such as a wound increases the difficulty of eradication through conventional antibiotic therapy. In this case, *C. nobile* was able to effectively inhibit the biofilm formation of strains isolated from wound infections, showing its potential value as an alternative anti-infection agent.

Chamomile is a potential source of anti-QS compounds for the development of effective alternative therapeutics. The findings of this study confirm the anti-QS activity of *C. nobile* in *P. aeruginosa*, and suggest that further research at the molecular level is warranted to explore the exact mechanisms of the action of these phytochemicals. The anti-QS property of chamomile may play an important role in antibacterial activity, and offers an additional strategy in the fight against bacterial infections. Indeed, the anti-bacterial concentration was 2-fold greater than the effective concentrations against biofilm formation.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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