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*Article*

# Anti-quorum sensing potential of ethanolic extracts of aromatic plants from Cyprus flora

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**Abstract:** Quorum sensing (QS) is a form of intra- and inter-species communication system which is employed by bacteria to regulate their collective behavior in a cell population-dependent manner. QS has been implicated in the virulence of several pathogenic bacteria. This work aimed to investigate the anti-quorum sensing (anti-QS) potential of ethanolic extracts of eight aromatic plants of Cyprus namely, *Origanum vulgare subsp. hirtum, Rosmarinus officinalis, Salvia officinalis, Lavendula spp., Calendula officinalis, Melissa officinalis, Sideritis cypria,* and *Aloysia citriodora*. We initially assess the effects of the extracts on autoinducer 2 (AI-2) signaling activity, using *Vibrio harveyi* BB170 as a reported strain. We subsequently assess the effect of the ethanolic extracts on QS-related process including biofilm formation and swarming and swimming motilities of *Escherichia coli* MG1655. Of the tested ethanolic extracts those of *Origanum vulgare subsp. hirtum, Rosmarinus officinalis, and Salvia officinalis* were the most potent AI-2 signaling inhibitors while the extracts from the other plants exhibited low to moderate inhibitory activity. The three ethanolic extracts also inhibited the biofilm formation (>60%) of *E. coli* MG1655, as well as its swimming and swarming motility in a concentration-dependent manner. These extracts may consider true anti-QS inhibitors because they disrupt QS-related activities of *E. coli* MG1655 without affecting bacterial growth. The results suggest that plants from the unexplored flora of Cyprus could serve as a source to identify novel anti-QS inhibitors to treat infectious diseases caused by pathogens resistant to antibiotics.

**Keywords:** quorum sensing; autoinducer; organic plant extracts; biofilms; swimming motility; swarming motility

## 1. Introduction

Antibiotics have been widely used for the prevention and treatment of bacterial infections in both humans and animals. However, their inappropriate use has led to the development of multi-drug resistance pathogens [1]. On the contrary, the development/identification of new antibiotics has steadily decreased since the 1970s [2] while many pharmaceutical companies have abandoned research on antibiotics [3]. Thus, the Word Health Organization (WHO) has recently called antibiotic resistance "*an increasingly serious threat to global public health that requires action across all government sectors and society*" (*http://www.who.int/mediacentre/factsheets/fs194/en*). It is predicted that by 2050, 10 million deaths worldwide will be attributable to antimicrobial resistance [4]. Bacteria not only develop resistance to antibiotics very fast but also share it with other bacteria [5]. Amongst the various mechanisms of bacterial drug resistance, the most threatening is those that involve resistance genes that are on plasmids and integrons [6]. This kind of information is shared not only between individual bacteria of the same species but also between species and in several cases between bacterial kingdoms [7]. Interestingly, Gram-negative (G) bacteria can obtain antibiotic resistance genes from a shared pool [8].

Several bacteria regulate their behavior in a cell density-depend manner through a cell-to-cell signaling mechanism called quorum sensing (QS) [9,10]. This intercellular communication system regulates gene expression and is affected by bacterial cell population density. The signaling molecules used in QS are secreted at very low concentrations and

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are effective only when the bacteria reach a high population density [11]. QS bacteria produce and release acyl-homoserine lactone (AHL)-signaling molecules, also called autoinducers (AIs), that accumulate in the environment as the cell density increases [12]. When a threshold stimulatory concentration of AIs is achieved, a signal transduction cascade is initiated that ultimately is translated into a change in the behavior of the bacteria. The QS has been shown to play significant roles in the regulation of virulence factors in several pathogens as the release of AIs facilitates the transcription of specific genes involved in antibiotic resistance [13], biofilms formation [12,14], and swarming motility [15].

AHLs are biosynthesized by members of the LuxI family of AHL synthases and are mainly used by $G^-$ bacteria. Gram-positive ($G^+$) bacteria do not harbor LuxI or LuxR homologs and instead utilize modified oligopeptides as AIs. In addition, a "universal" quorum sensing signal, the autoinducer-2 (AI-2), encoded by the *luxS* gene, has been identified in both $G^-$ and $G^+$ bacteria. It has been demonstrated that the luxS-mediated quorum sensing (AI-2 signaling) is a universal communication system involved in the regulation of various behaviors in bacteria [16]. Importantly, AI-2 is employed for interspecies communication between $G^+$ and $G^-$ bacteria and thus is of particular interest [17]. The AI-2 is widely used as a target for the screening of potential anti-quorum sensing (anti-QS) compounds using the *Vibrio harveyi* bioassay as previously described [18,19].

Knowing the significance of QS during bacteria pathogenesis research has focused on inhibiting QS. In contrast to antimicrobial compounds, anti-QS or 'anti-pathogenic' compounds do not cause cell death or inhibition of growth [20]. It has been demonstrated that the use of anti-QS compounds leads to the decrease of bacterial pathogenicity and biofilm formations, whereas it enhances the susceptibility of bacteria to antimicrobial drugs (e.g., antibiotics) and bacteriophages [21]. Several plant extracts and essential oils, exhibit anti-QS activity because they have a similar structure to molecules that are essential for the QS communication system (e.g., AHL). Thus, these anti-QS molecules can inhibit the AHL activity by competing with them [22]. In addition, plant extracts are also able to degrade the signal receptors (e.g., LuxR/LasR) of the AHL molecules [23]. Other natural extracts employ a combined mechanism to inhibit QS signaling i.e. they interfere with AHL activity while they are able to reduce the synthesis of AHLs by the bacteria [24]. The anti-QS potential of natural compounds has been reviewed elsewhere [25,26] and will not be discussed here.

Cyprus is located in the extreme north-eastern corner of the Mediterranean Sea and consequently, both the soil and climatic conditions might contribute to the large variety in the plant chemotypes. The flora of Cyprus is rich in endemic taxa and comprises 1640 indigenous taxa (species and subspecies), 244 introduced taxa occurring in the wild, 42 hybrids, and 84 species with unclear status [15]. In addition, more than 650 medical plants have been identified in Cyprus. However, the antimicrobial properties of Cyprus medicinal and aromatic plants have not extensively been studied and therefore they offer an attractive repertoire of phytochemicals with novel microbial disease-controlling potential. These plants and their extracts can open up the possibility of identifying novel quorum sensing inhibitors.

In this context, this work aimed to evaluate the anti-QS potential of ethanolic extracts of eight plants namely *Origanum vulgare subsp. hirtum, Rosmarinus officinalis, Salvia officinalis, Lavendula spp., Calendula officinalis, Melissa officinalis, Sideritis cypria,* and *Aloysia citriodora* from Cyprus flora. The effect of extracts on AI-2 signaling activity of *E. coli* MG1655 was initially evaluated using the well-established *V. harveyi* bioassay [18]. The effects of extracts were further evaluated against other bacterial functions related to QS including biofilm formation as well as swimming and swarming motilities. The effects of the organic extracts on bacterial growth and viability were also investigated. To the best of our knowledge, this is the first study that examines the anti-QS properties of extracts obtained from plants of Cyprus flora.

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## 2. Results

### *2.1. Inhibition of AI-2 activity by the ethanolic extracts*

We initially evaluate the anti-QS potential of the ethanolic extracts of the eight plants by monitoring the AI-2 inhibition using the well-studied *V. harveyi* bioassay [18]. In detail, AI-2 inhibition was determined by incubating the *V. harveyi* BB170 reporter strain with a known concentration of exogenous AI-2 (i.e., cel-free supernatant-CFS from an *E. coli* culture) to induce luminescence and with either one of the ethanolic extracts or its respective blank medium (i.e., CFS without the extract). Inhibition is deemed to have occurred when the luminescence of the sample is lower than that of its respective blank.

It has been previously demonstrated that the AI-2 signal molecule of *E. coli* reaches its maximum concentration at the mid-to-late growth phase while a significant decrease in its concentration is observed at the stationary phase [27]. To investigate whether the release of AI-2 signaling molecule is growth-dependent we initially evaluated the levels of AI-2 production by *E. coli* MG1655 at various time points. *V. harveyi* BB120 was used as a positive control. AI-2 activity was expressed as fold activation compared to the non-inoculated- with- CFS medium (negative control). Figure 1 shows the fold induction of the supernatant collected from *E. coli* MG1655 grown in LB supplemented with 0.5% glucose, as measured during the *V. harveyi* bioassay. The concentration of the AI-2 signaling molecule of *E. coli* MG1655 increased with the incubation time until 6 h, however, a decrease was observed after 6 h of growth. Based on these findings for the subsequent experiments, we used the CFSs from *E. coli* cultures that were grown for 5 h at 37°C in the presence of 0.5% glucose.

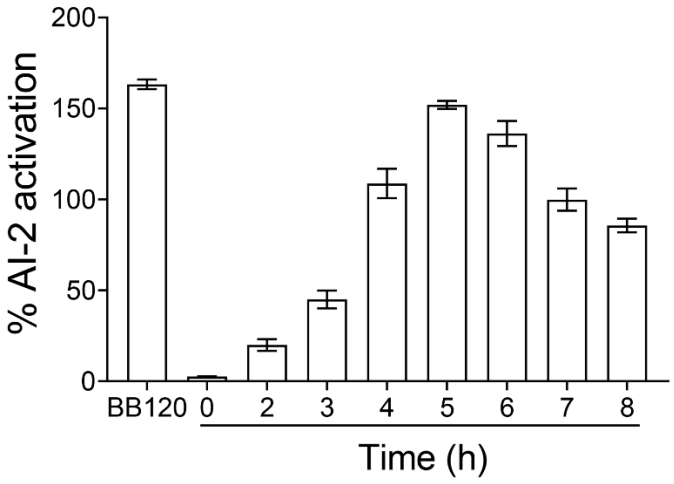


**Figure 1.** Time course of AI-2 signaling activity by *E. coli* MG1655**.** *E. coli* was grown in LB medium supplemented with 0.5% glucose at 37°C. At the indicated times, cell-free supernatants (CFSs) were prepared and assayed for AI-2 activity. The signaling activity is presented as the percent activation compared to the non-inoculated, with CFS, negative control. CFS obtained from an overnight culture of *V. harveyi* BB120 (AI-1$^+$, AI-2$^+$) was used as a positive control.

We subsequently tested the inhibitory effect of the ethanolic extracts from the 8 plants (Table 1) at a final concentration of 2 mg/mL on the AI-2 signaling activity.

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**Table 1** Plants used in this study and the recovery yields of their ethanolic extracts.

| Scientific name | Common name | Family | Extraction Yield (% dry mass) | Extract's color intensity [1] |
|---|---|---|---|---|
| *Origanum vulgare subsp. hirtum* | Oregano | *Lamiaceae* | 13.92 | + |
| *Rosmarinus officinalis* | Rosemary | *Lamiaceae* | 30.61 | + |
| *Salvia officinalis* | Common sage | *Lamiaceae* | 23.37 | +++ |
| *Lavendula spp* | Lavender | *Lamiaceae* | 9.25 | ++ |
| *Calendula officinalis* | Calendula | *Asteraceae* | 18.32 | ++ |
| *Melissa officinalis* | Lemon balm | *Lamiaceae* | 12.98 | ++++ |
| *Sideritis cypria* | Cyprian sideritis | *Lamiaceae* | 14.87 | ++++ |
| *Aloysia citriodora* | Lemon beebrush | *Verbenaceae* | 7.20 | +++ |

[1] *Color iintensity of the ethanolic extracts in DMSO: +: bright green; ++: green; +++ dark green; ++++: very dark green*

It should be noted that due to the intense color of the majority of the ethanolic extracts (Table 1) concentrations higher than 2 mg/mL interfere with the downstream assays, especially with the motility assays and the evaluation of their bactericidal activity which are described in the following paragraphs. Using *V. harveyi* BB170 as a reported strain, the extracts from oregano and rosemary inhibited AI-2 activity by 92.2 ± 1.6 % and 93.5 ± 1.2 % respectively (Figure 2A). Moreover, the extract from common sage inhibited AI-2 activity by 67.1 ± 3.3 % whereas the extracts of the other 5 plants exhibited AI-2 inhibition ranging from approximately 7% to 45% (Figure 2A). We further examined the effect of extracts that exhibited the highest AI-2 inhibition viz. oregano, rosemary, and common sage, by testing different concentrations ranging from 0.25 to 2.0 mg/mL, and as shown in Figure 2B, all ethanolic extracts exhibited inhibited AI-2 signaling activity in a concentration-dependent manner.

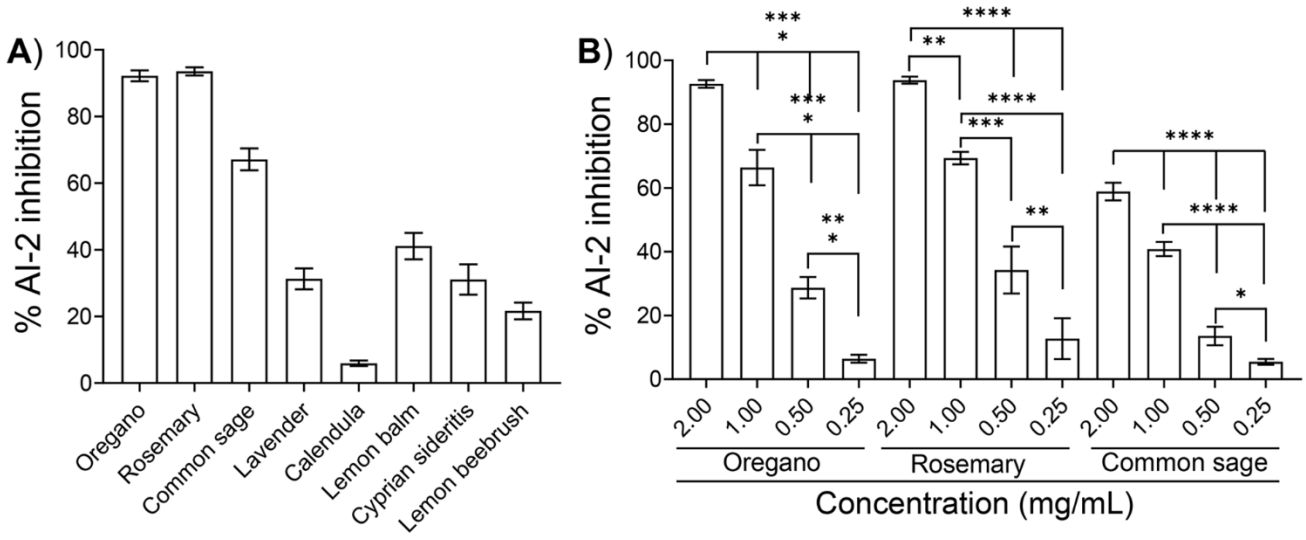


**Figure 2.** Effect of ethanolic extracts on AI-2 activity of *E. coli* MG1655**.** Cell-free supernatants were collected from an *E. coli* culture in LB medium supplemented with 0.5% glucose after 5h of cultivation and assayed for AI-2 activity in the presence of **A**) one of the indicated plant extracts at a final concentration of 2 mg/mL or **B)** different concentration of extracts obtained from oregano, rosemary or common sage. The signaling activity is presented as the percent inhibition compared to samples containing none of the extracts (blank control). Values are the means of the results of three independent experiments. Error bars indicate standard deviations. In **B)** ANOVA analysis followed by Tukey's multiple comparisons test was used for statistical analysis. Statistically significant differences are indicated with asterisks: $p$ *<0.05, ** $p$<0.01, ***<0.001 ****$p$<0.0001.

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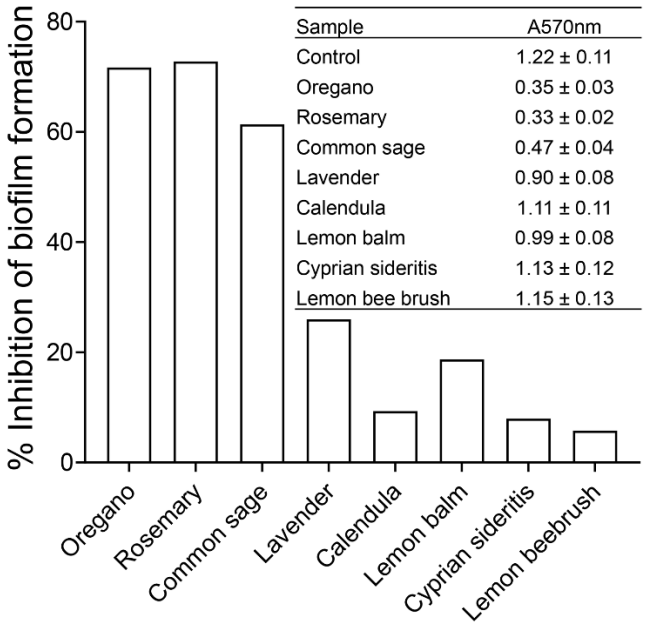
*2.2. Effect of plant extracts on biofilm formation*

It has been previously demonstrated that QS plays a vital role in biofilm formation and differentiation [28,29]. We, therefore, subsequently tested the effect of ethanolic extracts of oregano and rosemary at a final concentration of 1.0 mg/mL as well as of common sage at 2.0 mg/mL on the formation of *E. coli* biofilms using the crystal violet staining. It should be noted that for comparison purposes we selected the aforementioned concentrations of the three extracts because they produced a similar effect on AI-2 signaling activity (i.e., ~ 60% inhibition). The extracts of lavender, calendula, lemon balm, Cyprian siderites, and lemon beebrush were also tested at a final concentration of 2 mg/mL. The results are summarized in Figure 3 and as shown extracts from oregano, rosemary, and common sage exhibited the highest inhibitory effect (>60%) on biofilm formation of *E. coli* MG1655. Biofilm formation was lesser affected (26% and 19% respectively) by the ethanolic extracts obtained from lavender and lemon balm, while the extracts from calendula, Cyprian sideritis, and lemon beebrush had only a sight effect on biofilm formation by *E. coli* MG1655 (Figure 3).




| Sample | A570nm |
|---|---|
| Control | 1.22 ± 0.11 |
| Oregano | 0.35 ± 0.03 |
| Rosemary | 0.33 ± 0.02 |
| Common sage | 0.47 ± 0.04 |
| Lavender | 0.90 ± 0.08 |
| Calendula | 1.11 ± 0.11 |
| Lemon balm | 0.99 ± 0.08 |
| Cyprian sideritis | 1.13 ± 0.12 |
| Lemon bee brush | 1.15 ± 0.13 |

**Figure 3.** Effect of the ethanolic extracts from the eight aromatic plants from Cyprus flora on biofilm formation by *E. coli* MG1655 as quantified by crystal violet staining and measuring at A570nm. Data are presented as the percentage of biofilm formation compared to the control containing none of the ethanolic extracts**.** Inset: Absorbance values at 570 nm ± SD following the crystal violet staining of three independent experiments

*2.3 Impact of phytochemicals on swarming and swimming motility of E. coli MG 1655*

QS-mediated swarming and swimming motilities are important features of G-negative bacteria for the surface attachment during the early stages of biofilm formation and the subsequent maturation of biofilm [30]. We, therefore, investigated the effect of the eight ethanolic extracts on the motility of *E. coli* MG1655.

The results are summarized in Figure 4 and as shown the ethanolic extracts of oregano, rosemary, and common sage significantly reduced both types of motilities. Importantly, these three ethanolic extracts inhibited both types of motilities in a concentration-dependent manner (Supplementary Figure S1). The ethanolic extracts of the

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other five plants had either a slight or no effect on both types of motilities of *E. coli* MG1655 (Figure 4).

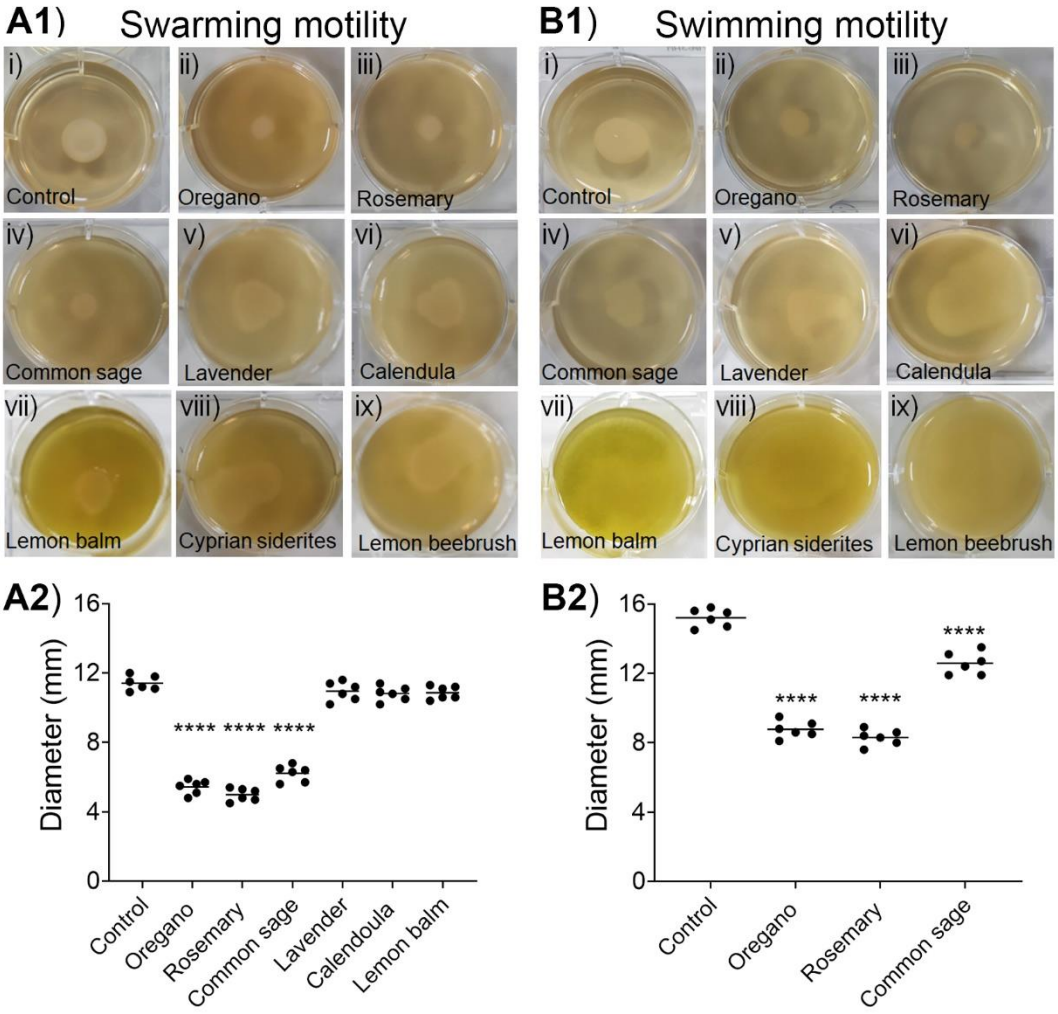


**Figure 4.** Inhibition of swarming (A1 and A2) and swimming (B1 and B2) motilities *of E. coli* MG1655 by the ethanolic extracts**.** In panels (ii, iii) and panels (iv to ix), *E. coli* MG1655 was grown on LB agar containing 1 mg/mL or 2 mg/mL, respectively of the indicated ethanolic extract. In A2 and B2, results are shown as mean ± SD of six independent experiments. Horizontal bars indicate mean values. ANOVA analysis followed by Tukey's multiple comparisons test was used for statistical analysis. Only statistically significant differences are shown and indicated with asterisks: ****$p$<0.0001

In detail, at the highest concentrations tested the ethanolic extracts of oregano and rosemary (both at 1mg/mL) as well as of common sage (2mg/mL) significantly ($p$<0.0001) inhibited the swarming motility of *E. coli* MG1655 by 54.7%, 58.3%, and 48.4 %, respectively. The ethanolic extracts of lavender, calendula, and lemon balm had only a limited effect on swarming motility (~10% inhibition), but these differences did not reach statistical significance. On the other hand, we could not evaluate the effect of the ethanolic extracts of Cyprian siderites and lemon beebrush on the swarming motility of *E. coli* MG1655 because diffused zones were observed. In terms of inhibition of swimming motility, the highest inhibition (45.7 %; $p$<0.001) was recorded in the presence of the ethanolic extract of rosemary at a final concentration of 1mg/mL. The ethanolic extracts of oregano (1 mg/mL) and common sage (2 mg/mL), inhibited the swimming motility of *E. coli* MG1655 by 42.3% ($p$<0.0001) and 17.2 % ($p$<0.0001) respectively. The effect of the ethanolic extracts of the other five plants on the swimming motility of *E. coli* MG1655 could not be evaluated because huge diffused zones were formed. Inhibition of both types of

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motilities by the ethanolic extracts oregano, rosemary, and common sage can be correlated with the reduced ability of *E. coli* to form biofilms in the presence of the aforementioned ethanolic extracts.

### *2.4. Effect of plant extracts on biofilm formation*

To verify that any of the observed anti-QS activities of the ethanolic extracts from oregano, rosemary, and common sage were not connected to bactericidal activity, the effect of the extracts on the growth of *E. coli* MG1655 was examined. Interestingly, none of the three ethanolic extracts displayed any bactericidal activity, as determined by the inhibition of growth assay and viable plate counts (Supplimentary Figure S2). Therefore, the ethanolic extracts from oregano, rosemary, and common sage can be considered to be true QS inhibitors that do not rely upon antibacterial activity as traditional antibiotics [31].

## 3. Discussion

Antibiotics, due to their bactericidal effect, play an important role in both the prevention and treatment of bacterial infections [32]. Unfortunately, bacteria under selective pressure from antibiotics have developed sophisticated mechanisms to fight these drugs, leading to the development of strains resistant to antibiotics [33]. The development of resistance to antibiotics by pathogenic bacteria is a major health issue worldwide (reviewed in [34]). To this end, the attention of the scientific community has been turned to the identification of antipathogenic drugs which do not kill bacteria and thus do not impose selective pressure on the development of resistance strains[35]. Thus, it could be possible to inhibit the virulence of *pathogenic bacteria* without killing cells; while such antipathogenic compounds may be used alone or in combination with antibiotics [36].

In the past years, it has been demonstrated that both $G^-$ and $G^+$ bacteria use QS to coordinate gene expression in a cell density-dependent manner [37]. When bacteria reach a critical concentration they release signal molecules which are called AIs [36].QS is often employed to regulate genes that are particularly useful when expressed by a bacterial community, including genes that are implicated in virulence, biofilm formation, swarming and swimming motility, stress resistance, and resistance to antibiotics [38,39]. Therefore, inhibition of QS communication between bacteria could be used as an alternative strategy to fight multidrug resistance bacteria while any compound able to inhibit AI activity without growth inhibition can be considered to be a promising QS inhibitor [36]. It has been demonstrated that several plant extracts and essential oils exhibit antimicrobial and anti-QS activity and therefore, identifying anti-QS compounds from natural sources including aromatic plants is of particular interest in the scientific community[40]. The essential oils of several plants have demonstrated promising anti-biofilm formation and anti-QS activities [41].

In this work, the anti-QS activity of the ethanolic extracts of eight aromatic plants from Cyprus flora (Table 1) was examined. Screening of compounds for anti-QS activity was carried out using a variety of bioassays, including inhibition of AI-2 signaling activity, inhibition of biofilm formation, and motility assays. Screening of ethanolic extracts for inhibition of AI-2 activity was performed using the widely used *V. harveyi* assay using BB170 as a reported strain. *V. harveyi* BB170 is exquisitely sensitive to AI-2 (it has the QS phenotype AI-$1^-$, AI-$2^+$), and therefore, even low amounts of AI-2 can be detected using this bioassay. Inhibition was considered when the luminescence of a tested compound (i.e., ethanolic extract) is lower than the respective blank control. Our preliminary results revealed *E. coli* MG1655 exhibits significant AI-2 activity in LB supplement with 0.5 after 5 h cultivation while the signaling activity was comparable to that of *V. harveyi BB120 (*AI-$1^+$/AI-$2^+$*)* (Figure 1). It should be noted that when *E. coli* is grown in LB supplemented with glucose, the glucose prevents the uptake of AI-2 into the cell; hence, it accumulates in the supernatant[42,43]. Subsequently, our preliminary screening revealed that the ethanolic extracts of oregano and rosemary at 2mg/mL exhibited the highest inhibition of AI-2

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activity (>90%) (Figure A). Importantly, the ethanolic extracts of the three plants had a concentration-dependent effect on AI-2 signaling activity (Figure 2B). Previous studies have highlighted the anti-QS potential of extracts and essential oils and/or other single bioactive compounds of oregano [25,44-46] and rosemary [47,48]. Despite the antimicrobial properties of extracts of common sage (*Salvia officinalis*) that have been previously reported [49], their anti-QS potential remains inconclusive. Our initial screening revealed that ethanolic extract of common inhibited AI-2 activity in a concentration-dependent manner (Figure 2B), while at the highest concentration tested (2 mg/mL) a 65% inhibition of AI-2 activity was recorded (Figure 2A and 2B). Unfortunately, due to the intense color of the ethanolic extract of common sage (Table 1) concentrations higher than 2 mg/mL interfere with the motility assays and growth inhibitions assays while precipitation of the extracts was observed at concentrations ≥ 5 mg/mL in aqueous solutions probably due to their hydrophobic nature. In terms of inhibition of AI-2 signaling activity of the ethanolic extract of lavender, calendula, lemon balm, Cyprian sideritis, and lemon inhibited the AI-2 activity by less than 45% (Figure 2A).

We evaluated further the anti-QS activity of the eight extracts (Table 1) using concentrations that produce comparable AI-2 inhibition (~ 60%) i.e., 1mg/mL for oregano and rosemary and 2 mg/mL for common sage. The ethanolic extracts of the other 5 plants were also tested at 2 mg/mL. We subsequently tested the effect of the 8 ethanolic extracts on the formation of biofilms by *E. coli* MG1655 using the crystal violet staining. QS has been implicated in the development of biofilms in both $G^-$ and $G^+$ species while biofilm formation is one of the strategies employed by bacteria for developing resistance to antibiotics [50]. Also treating diseases caused by bacteria that form biofilms required prolonged treatment that may also lead to antibiotic resistance due to high evolutionary pressure [51]. Herein the extracts of oregano, rosemary, and common sage significantly inhibited the formation of biofilms by *E. coli* MG1655 (Figure 3) without affecting the bacteria growth (Supplementary Figure 2). The ability of extracts and essential oils of oregano to inhibit the formation of biofilms formed by *Candida spp. [52], Staphylococci, E. coli [53]* have been previously reported. Likewise, the inhibitory effect of extracts of rosemary on biofilm formation by a variety of pathogenic bacteria has been Candida albicans, Staphylococcus aureus, and *Pseudomonas aeruginosa* has been described [54]. Recently, Selim, et al [55] reported the antibiofilm potency of the essential oil of common sage (*Salvia officinalis* L). against antibiotic-resistant *Salmonella enterica.* The potential of extracts of common sage to inhibit biofilm formation by *P. aeruginosa* has also been reported [56].

QS-dependent swimming motility driven by flagella is important for the initiation of cell/surface attachment during biofilm formation [57]. In this work we demonstrated that the extracts of oregano, rosemary, and common sage inhibit the swimming motility of *E. coli* MG1655 in a dose-dependent manner (Figure 4 and Supplementary Figure 1). In addition to the swimming migration swarming motility, another QS-dependent motility, has been implicated in biofilm formation [58]. Our results, revealed a dose-dependent inhibition of the swarming motility of *E. coli* MG1655 by the extracts obtained from the three aforementioned plants (Figure 4 and Supplementary Figure 1). The correlation of inhibition of biofilm formation with the reduced swimming and swarming motility of a variety of bacteria pathogens in the presence of different extracts of plants and fruits including *Capparis spinosa* [59] and *Salvadora persica* L. [60] as well as in the presence of clove oil has been reported [61]. To the best of our knowledge, this is the first study that examines the effects of extracts of oregano, rosemary, and common sage on both types of motilities of *E. coli* MG1655.

## 4. Materials and Methods

### *4.1 Selection and preparation of plants*

A total of 8 plants (Table 1) were collected from the Cypriot National Agricultural Department, Nicosia, Cyprus. Selection and collection of plants were carried out based on good plant authentication and identification practices (GPAIP) and good agricultural and collection practice (GACP) [62]. The collected plants were handled with standard storage

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protocols and transported by being wrapped in plastic bags. The plants were washed thoroughly under running tap water, rinsed with double distilled water, air-dried at room temperature (~25°C) under shade, cut to the appropriate size, packed in plastic bags, and kept until extraction.

*4.2 Extraction of active compounds from plants*

Extraction techniques have mainly focused on finding solutions that minimize the use of solvents and enable process intensification for the cost-effective production of high-quality extracts. Directive REACH (Registration, Evaluation, Authorisation, and Restriction) limited the use of several chemical solvents and reagents, in extraction or industrial manufacturing products (https://echa.europa.eu/regulations/reach/understanding-reach). In this work, we followed the "Six Principles of Green Extraction of Natural Products"[63] which are: 1: Innovation by the selection of varieties and use of renewable plant resources; 2: Use of alternative solvents and/or water or agro-solvents; 3: Reduce energy consumption by energy recovery and using innovative technologies; 4: Production of co-products instead of waste; 5: Reduce unit operations and favor safe, robust and controlled processes; 6: Aim for a non-denatured and biodegradable extract without contaminants.

Based on the aforementioned principles, we used ethanol as a solvent for the extraction of phytochemicals, and instead of using the traditional and energy and time-consuming Soxhlet methodology, we used the sonication-assisted technology which saves time and reduces energy consumption. Our preliminary experiments revealed that the best ratio (dry plant/volume) to obtain the maximum extract per dry mass was 1g per 20 mL of solvent. Lower quantities i.e., 0.5 g /10 mL solvent did not result in similar quantities, demonstrating that there is a cut-off in the method. As a result, 1g of dry material (plant) was placed in a 50 mL centrifuge vial. 20mL of ethanol was added and the mixture is sonicated in an ultrasonic water bath (Grant, UK) for 45min at 45°C and 200W, 32-38KHz. Subsequently, the solution was filtered through a 0.25 μm filter and the solvent was removed under vacuum at 45°C in rotavapor Buchi R-210. The extractions yields are summarized in Table 1. The residual extracts were resuspended in DMSO previously filtered with a 0.2 μM syringe filter (VWR, West Chester, PA).

*4.3 Bacterial strains, media, and culture condition*

*Escherichia coli* MG 1655 (ATCC-700926), *Vibrio harveyi* BB-120 (ATCC-BAA-1116), and *V. harveyi* BB-170 (ATCC-BAA-1117) were purchased from American Type Culture Collection (ATCC; Wesel, Germany).

*E. coli* was grown in Luria Bertani (LB) medium consisting of 1% tryptone, 0.5% yeast extract, and 1% NaCl at 37°C. *V. harveyi* BB-120 and BB-170 were grown at 30°C in autoinducer bioassay (AB) medium (ATTC medium: 2034) consisting of (per 1L) 17.53 g NaCl, 6.02 g $MgCl_2$, 2.0 g casamino acids (vitamin-free). The pH of the medium was adjusted to pH 7.0 with 1 M KOH and subsequently, the medium was autoclaved at 121°C for 15 min. The solution was cooled to room temperature and 10 mL of 1 M potassium phosphate buffer, pH 7.0, 10 mL of 0.1 M sterile arginine solution, and 20 mL of 50% sterile glycerol were added to the medium.

*4.4 Autoinducer-2 bioassay*

The AI-2 bioassay was carried out as previously described [64,65]. The assay is based on the ability of the reported stain *V. harveyi* BB170 to specifically bioluminate in response to AI-2. At lower cell densities of BB170 ($10^6$–$10^7$ CFU/mL), the bioluminescence can be detected in response to the added AI-2 [66].

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*4.4.1 Preparation of cell-free supernatants*

*E. coli* was grown overnight at 37°C in LB medium supplemented medium containing 0.5 % glucose. The next day, the overnight culture was used to inoculate (1:100) fresh LB medium containing 0.5 % glucose, and the cultures were incubated at 37°C for various times as indicated in the text under continuous shaking at 250 rpm. Cell-free supernatants (CFSs) were prepared by centrifuging the culture at 16,000g for 15 min at 4 °C, and the resulting supernatants were passed through 0.2-µm syringe filters (VWR, West Chester, PA), aliquoted and stored at −20 °C until AI-2 bioluminescence assay was carried out. CFSs containing *V. harveyi* AI-2 were prepared from *V. harveyi BB120* (AI-1$^+$, AI-2$^+$) and used as positive controls. In brief, *V. harveyi* BB120 was grown overnight at 30 °C in AB medium under continuous shaking. CFSs we recovered from the overnight culture as described above for *E. coli*.

*4.4.2 Inhibition of AI-2 by the ethanolic extracts*

The *V. harveyi* reported strain BB170 was grown for 16 h at 30 °C in AB medium and subsequently diluted (1:5000) into fresh AB medium. 90 µL of the diluted cells were added into the wells of a 96-well plate and mixed with either 10 µL of *E. coli* MG1655 or *V. harveyi* BB120 (for the screening experiments) or 9 µL of CFSs of *E. coli* MG1655 and 1 µL of each of the ethanolic extracts (of various concentrations as described in the Results). In addition, blank controls (9µL of CFSs + 1 µL DMSO) and negative control (9 µL AB medium + 1 µL DMSO) were included in each experiment.

The plates were incubated at 30 °C under continuous shaking (100 rpm) and luminescence readings (in relative light units/RLU) were recorded every 20 min using a Perkin Elmer VictorX3 2030 Multiplate reader, (PerkinElmer, Waltham, MA) in the chemiluminescence mode. The inhibition of AI-2 activity was expressed as a percentage of the relative to the blank control and calculated using the following equation (eq. 1) [67]:

$$\%\textbf{AI2 inhibition} = \left(1 - \frac{\textbf{RLU of sample}}{\textbf{RLU of blank control}}\right) \times 100 \quad (1)$$

*4.5. Inhibition of biofilm formation*

The effect of plant extracts on biofilm formation was assessed in sterile 96-well flat-bottom polystyrene plates as previously described [68] with some modifications. Positive controls (bacteria cells + LB), medium controls (LB only), and solvent controls (cells + LB + DMSO) were used. All experiments were carried out in triplicates.

The appropriate concentration of plant extract was added to the test wells before inoculation. Plates were incubated at 37 °C under continuous shaking (100 rpm). After 48 h of cultivation, the content of each well was discarded, rinsed 3 times with phosphate-buffered saline (PBS), and fixed by drying for 1 h at 37 °C in the incubator. When the wells were fully dry, 200 µL of 0.1 % crystal violet stain were added to each well and incubated for 15 min at 25°C. The excess dye was rinsed off using tap water and subsequently, 200 µL of 9 6% ethanol was added to the wells. The stain adhering to the biofilm biomass was pulled off with the ethanol and transferred to clean 96-well plates and the absorbance at 570 nm (A570nm) was measured in a Perkin Elmer VictorX3 2030 Multiplate reader (PerkinElmer, Waltham, MA). The biofilm inhibition rate was defined using the following equation (eq.2):

$$\%\textbf{Biofilm inhibition} = \left(1 - \frac{\textbf{A570nm of the samle}}{\textbf{A570nm of the positive control}}\right) \times 100 \quad (2)$$

All crystal violet assays were run in triplicate, with a minimum of three replicates per assay.

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*4.6 Motility assays*

Swimming and swarming motility assays were performed as previously described [59,69] with some modifications. Both swimming and swarming motility assays in the wells of 6 well-plates (5 mL per well). Overnight cultures (2 µL; ~$10^7$ CFU/mL) of *E. coli* in LB medium were point inoculated in swarming agar consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl and 0.5% w/v agar with different concentrations of the ethanolic extracts as described in the "*Results*" section. To assess the effect of ethanolic extracts on the swimming motility of *E. coli* 2 µL (~$10^7$ CFU/mL) from an overnight culture of the bacterium were point inoculated at the center of the wells of 6-well plates containing 1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.3% (w/v) agar containing one of the ethanolic extracts at a final concentration of 1.0 mg/mL or 2.0 mg/mL. In both the swimming and swarming motility wells containing none of the extracts were used as controls. Plates were incubated at 37°C in the upright position for 16h. The swimming and swarming migrations were recorded by measuring the diameter of swim zones or swarm fronts, respectively of the bacterial cells after the incubation periods.

*4.7 Effect of ethanolic extracts on bacteria growth*

The effect of the ethanolic extracts on *E. coli* MG1655 growth was evaluated in liquid culture (200 µL) in the wells of a 96-well plate. Serial dilutions were performed to examine the effect of ethanolic extracts of oregano, rosemary, and common sage at final concentrations ranging from 0.5 – 2 mg/mL after 20 h of cultivation. Growth controls (bacteria cells + LB), medium controls (LB only) and solvent controls (cells + LB + DMSO) were used. All experiments were carried out in triplicates. Optical density values at 600 nm ($OD_{600nm}$) were obtained using a Perkin Elmer VictorX3 2030 Multiplate reader, (PerkinElmer, Waltham, MA) at 0 h and 20 h post-inoculation. To account for the effect of extract color (bright green to very dark green) on the $OD_{600nm}$ the following formula (eq. 3) was used [70]:

$$\%\ \mathbf{inhibition} = \left(\mathbf{1} - \left(\frac{\mathbf{OD_{t20h} - OD_{t0h}}}{\mathbf{OD_{gc20h} - OD_{gc0h}}}\right)\right) \times \mathbf{100} \qquad (3)$$

where: $OD_{t20h}$ is optical density (600 nm) of the test well at 20 hours post-inoculation; $OD_{t0h}$ is optical density (600 nm) of the test well at 0 hours post-inoculation; $OD_{gc20h}$ is optical density (600 nm) of the growth control well at 20 hours post-inoculation, and $OD_{gc0h}$ is optical density (600 nm) of the growth control well at 0 hours post-inoculation.

The effect of the ethanolic extracts of oregano, rosemary, and common sage on bacterial growth was further assessed by performing viable plate counts as previously describe [69] with some modifications. Ethanolic extracts at a final concentration of 1 mg/mL or 2 mg/mL and cultures of *E. coli* ($10^7$ CFU/mL) were added to the wells of a 96-well plate (200 µL per well). Untreated bacteria were included in control wells. The plates were incubated at 37 °C, without shaking for 24 h. At the end of this period, bacterial suspensions were transferred from the wells to Eppendorf tubes, centrifuged at 5000g for 5 min at 4 °C, washed three times with PBS, and resuspended in 200 µL of fresh LB medium. Each suspension was subsequently serially diluted in LB and plated on LB agar. The number of viable bacteria was determined after incubation at 37 °C for 16h. Results were expressed as CFU/mL.

*4.8 Statistical analysis*

Unless otherwise stated, experiments were carried out in triplicate and the data are presented as mean values ± standard deviation (SD). One-way ANOVA analysis followed by Tukey's multiple comparisons test was used for statistical analysis. Statistical significance was set at $p<0.05$. Statistical analysis was performed using GraphPad Prism (v.8.2, GraphPad Software Inc., San Diego, CA, USA).

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## 5. Conclusions

The trend of using natural compounds as anti-QS is gradually becoming an attractive approach in the field of developing new drugs to fight antibiotic-resistant bacteria. In this work, we identified three ethanolic extracts from endemic plants of Cyprus that significantly inhibit AI-2 signaling activity. The AI-2 molecule is of particular interest because is a universal interspecies signaling molecule. Thus, inhibition of AI-2 could be a potential strategy to control bacterial pathogenicity. Biofilm formation which is also controlled by QS is one of the biggest challenges for human/animal health, as well as for the food industry. The three extracts also inhibited the formation of biofilm in *E. coli* MG1655 as well as the swimming and swarming motility of the bacterium. Several natural products including organic extracts of aromatic plants display promising anti-QS activities by preventing biofilm formation, and bacteria motility and thus they could reduce the virulence and pathogenicity of antibiotic resistance bacteria. These extracts identified in this work could be served as a starting point for further optimization and identification of novel anti-QS agents for the treatment of biofilms. To conclude, in this work, we identified a pool of potential anti-QS inhibitors that do not affect bacterial growth. Importantly, the identification of anti-pathogenic compounds" i.e., molecules that reduce the virulence of bacteria without killing do not impose selective pressure for the development of resistant strains. Further experiments and analysis of the composition of the extracts in terms of bioactive compounds are required to elucidate the mechanism(s) by which they inhibit the QS activity in bacteria.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Inhibition of swarming and swimming motilities of E. coli MG1655 by different concentrations of the ethanolic extracts; Figure S2: Effect of the ethanolic extracts of oregano, rosemary, and common sage on *E. coli* MG1655 growth.

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