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Antibiofilm, antiquorum sensing and antioxidant activities of *Calendula officinalis* L., *Hypericum perforatum* L. and *Trachystemon orientalis* (L.) G. Don essential oils

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Bacteria can coordinate biofilm formation through antiquorum sensing and create a more effective defense mechanism. Therefore, strategies that inhibit biofilm formation and target the antiquorum sensing mechanism are needed to combat antibiotic resistance. This study was conducted to determine the antibiofilm and antiquorum sensing (anti-QS) effect of essential oils (EOs) obtained from *Calendula officinalis* L., *Hypericum perforatum* L. and *Trachystemon orientalis* (L.) G. Don. and also to calculate oxidative stress indices (OSI) by analysing oxidant and antioxidant capacity. Antibiofilm capacities of EOs against *Escherichia coli* were investigated by microplate method. Anti-QS activities were evaluated with the agar well diffusion test. The oxidant capacities of plant EOs, whose antioxidant value was determined with the Total Antioxidant Status kit (TAS), were also examined with the Total Oxidant Status kit (TOS), and the OSI were calculated from the ratio of the two. EOs of all three plants have strong antibiofilm and anti-QS effects. *T. orientalis*, which has 19 mm+ anti-QS effect, shows 93.11% biofilm inhibition effect and draws attention together with the other two plants used pharmacologically. EOs with low OSI levels are expected to have high phytotherapeutic efficacy. As expected, the EOs used are considered important components for their capacity to inhibit biofilm formation and the QS mechanism. This study constitutes the first step in using EOs as alternative agents for strategies that prevent the anti-QS mechanism and biofilm formation.

Keywords: Biofilm, *Chromobacterium violaceum* ATCC 12472, Oxidative stress index

Biofilm formation allows for a high rate of horizontal gene transfer of bacteria. This situation provides a chance for the transfer of antibiotic resistance genes between species, making the development of resistance inevitable[1,2]. Many physiological features such as biofilm formation and virulence expression of bacteria are regulated by the intercellular communication mechanism known as quorum sensing (QS) in bacteria[3]. Anti-QS agents support the antimicrobial process thanks to their ability to reduce virulence and block biofilm formation without the development of resistance[4]. The low cytotoxic effects of EOs and their anti-QS effect by mimicking QS molecules have paved the way for their design as therapeutic agents[5]. Similarly, plant EOs have high free radical scavenging effects, thanks to the phenolic compounds and terpenes they contain[6]. It has been proven that EOs with high antioxidant capacity can prevent cellular damage caused by reactive oxygen species by reducing oxidative stress[7].

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Trachystemon orientalis (L.) G. Don is widely available in the Caucasus, Eastern Bulgaria, Turkey and is especially widespread in the Black Searegion of Turkey. It is a plant, known as urination, blood purifying effects. This plant contains of tannins, essential oil, nitrate salts, mucilage, saponins andresin, cholineand β-sitosterol[8]. *Calendula officinalis* L. is widespread in various parts of the world and is used medicinally in Europe and China[9]. *C. officinalis* is utilized for its numerous beneficial properties, including anti-inflammatory, antioxidant, antitumor, antimicrobial, antidiabetic, and wound healing effects. This plant is rich in various compounds such as flavonoids, coumarins, carotenoids, glycosides, sterols, fatty acids, triterpenoids, saponins, amino acids, steroids, and quinones[10,11]. *Hypericum perforatum* L. is a perennial herbaceous plant native to Europe, Western Asia, and North Africa[12]. The oil extracted from *H. perforatum* has been used in traditional medicine for the treatment of various diseases such as cancer, heart disease, ulcer, gastritis, hemorrhoids and urogenital inflammations[13,14].

This study tries to determine to what extent plant essential oils can reduce biofilm formation on biofilm-forming Gram negative *E. coli* culture and how they affect QS communication. In addition, antioxidant and oxidant capacities were determined and the OSI value was calculated.

Materials and Methods

Preparation of bacterial culture

Biofilm forming *E. coli* strain was used to determine antibiofilm effects of plant EOs. It was suspended in 5 mL Mueller Hinton Broth (MHB) (Merck; Darmstadt, Germany) containing 0.5% glucose in sterile universal bottles. It was incubated at 37°C for 24 h before proceeding to the next steps. Experiments were performed by adjusting the culture turbidity of the microorganism to Mc Farland 0,5 (10^8 cfu/mL). The model organism *Chromobacterium violaceum* ATCC 12472 was used for the anti-QS experiment.

Preparation of essential oils

Three plant species, *C. officinalis*, *H. perforatum* and *T. orientalis* were used in this study. The plant samples were collected from natural habitats in Düzce province between April and June 2022 (*C. officinalis* [40°54'16.0"N 31°10'37.0"E] *H. perforatum* [40°54'23.0"N 31°11'13.0"E], *T. orientalis* [40°45'23.0"N 31°02'26.0"E]) and identified by Dr. Mustafa Eray Bozyel. Plant samples were dried in the shade at room temperature of 24°C. The EOs of three species were prepared by hydrodistillation in a Clevenger apparatus (Termal, Turkey), according to the method of Wang *et al*.[15]with minor modifications. Following extraction, ether was used to separate the water and oil, and then anhydrous Na_2SO_4 (Merck; Darmstadt, Germany) was used to remove the water and filtered. A pure EOs were obtained in a rotary evaporator under 150 rpm pressure at an average temperature of 20-24°C. By hydrodistillation of plant materials, oils were obtained with a yield of 0.60%, 0.24% and 0.12% on a dry weight basis for *C. officinalis*, *H. perforatum* and *T. orientalis*, respectively. After which the EOs was kept at +4°C in a refrigerator[15,16]. Before the analysis, three EO samples were freshly prepared with DMSO (Merck; Darmstadt, Germany) at the rate of 4, 8, 16 and 20% (w/v).

Antibiofilm assay

The potential of plant EOs to inhibit biofilm formation produced by the *E. coli* strain was evaluated using the microplate biofilm test[17,18]. First, 100 µL of sterile MHB was placed in the wells to be studied in a sterile 96 well microtiter plate. About 50 µL of plant EO in 4 different concentrations (4%, 8%, 16% and 20% (w/v) prepared with DMSO) was added to it. Finally, 50 µL of *E. coli* cells were inoculated. As a positive control, only *E. coli* cells were inoculated into sterile medium. Only sterile medium was used as negative control. After 48h of incubation at 37°C, planktonic cells were washed three times with 200 µL of phosphate buffered saline (PBS) and then allowed to dry for 20 min at 65°C. Biofilms were stained with 200 µL of 0.03% crystal violet (Merck; Darmstadt, Germany) for 15 min and then rinsed three times with distilled water. After the dye dried at room temperature was dissolved with 95% ethanol (Merck; Darmstadt, Germany), the OD was measured at 595 nm. The average absorbance of the three replicated samples was determined. Percent inhibition values were calculated for each EO concentration with the following Formula 1:

$$[(OD_{positive\ control} - OD_{treatment}) / OD_{positive\ control}] \times 100$$

Anti quorum sensing assay

In addition to anti biofilm screening of oils, anti-QS activity was qualitatively screened with the Agar Well Diffusion Test[19]. *C. violaceum* ATCC 12472 was incubated in Luria-Bertani Broth (LBB) (Merck; Darmstadt, Germany) for 16-18h in an orbital incubator operating at 28ºC and 150 rpm. Cultures were then adjusted to 0.5 McFarland standard (equivalent to 1 x 10^8 cells/mL). *C. violaceum* ATCC 12472 (10 mL) was inoculated into LB agar (1000 mL), poured into petri dishes and solidified. Wells were made in LBA medium, four in each petri dish, using a cork borer. 20 µL of plant EO at 4 different concentrations (4%, 8%, 16% and 20% (w/v)) prepared with DMSO was added to the wells. Petri dishes were incubated at 35°C for 24h. As a result of incubation, the opaque zones formed around the wells were evaluated as QS inhibition effect and the zones were measured in millimeters. This experiment was performed three times and the results were evaluated as the average of the three.

Antioxidant and oxidant capacity

Oxidative stress parameters of plant EOs were measured using Rel Assay TOS and TAS kits (Assay Kit Rel Diagnostics, Turkey) and OSI values were calculated. Kits were used according to the protocols specified by the manufacturer. Trolox was used as the

calibrator in the TAS kit and its value was calculated as mmol Trolox eq/L. Hydrogen peroxide was used as the calibrator in the TOS kit and its value was calculated as μmol H_2O_2 eq/L. In the determination of the oxidative stress index, the units of TOS value and TAS value were proportioned, as shown in Formula 2 and OSI values were calculated[16,20].

$$OSI\ (AU) = [TOS\ (\mu mol\ H_2O_2\ eq/\ L) / TAS\ (mmol\ Trolox\ eq / L)] \times 10$$

Statistical analysis

The study used triplicate for all tests conducted, and the obtained data are expressed as the mean ±standard deviation (SD) of the triplicate. The data of the antibiofilm and anti-QS experiments were analysed for statistical significance in SPSS Statistics 27.0.1 program. P <0.05 value was considered statistically significant compared to the control was determined for each data using One-way ANOVA followed by Dunnett's multiple comparison tests for mean with 95% confidence limit.

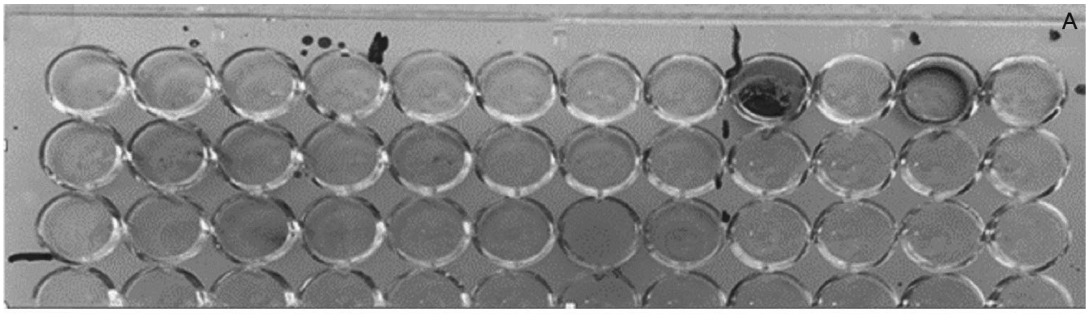
Results

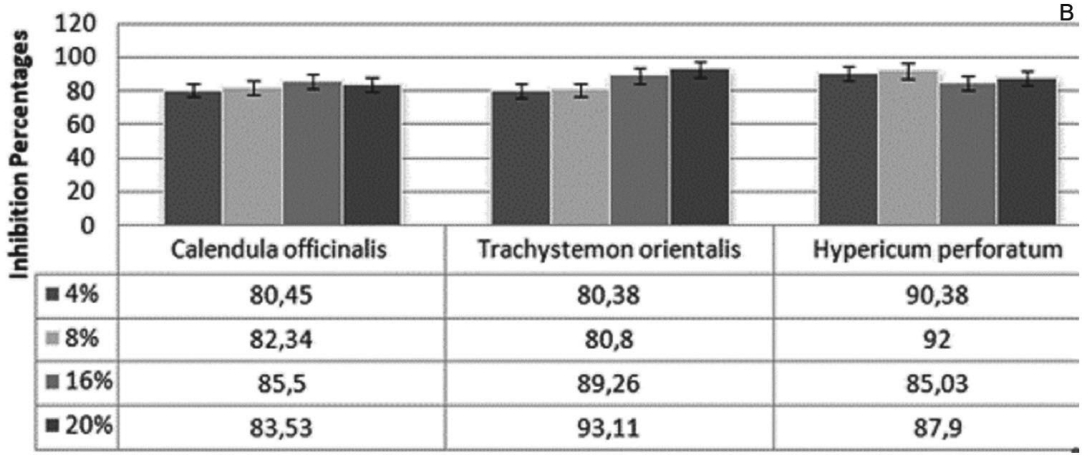
Anti biofilm activity of the essential oils

The inhibition effects of plant EOs on the biofilm formed by *E. coli* were investigated. Percent inhibition values were calculated for each EOs concentration by Formula 1 previously indicated. As shown in Fig. 1, four different concentrations of EOs affected biofilm formation at different levels. *C. officinalis* oil showed the highest inhibition effect at 8% concentration and prevented biofilm formation at 92.34% levels. As *T. orientalis* oil concentrations increased, biofilm inhibition increased and the highest inhibition percentage reached 93.11% at 20% concentration. *H. perforatum* oil, on the other hand, prevented biofilm formation at 8% concentration and 92%.

Anti quorum sensing activity of the essential oils

The loss of purple pigment in *C. violaceum* ATCC 12472 indicates the anti- QS effect of the bioactive components[21]. In current study, it was observed that plant EOs (due to the formation of an opaque zone without the formation of a transparent zone) showed QS inhibition activity without stopping the growth. *T. orientalis* EO showed the highest effect. The 19.33 mm QS inhibition of the oil studied at 4 different concentrations draws attention. This is followed by the EO of *C. officinalis*, which has an effect value of over 15 mm at each concentration. Among the EOs, the lowest value belongs to the 4% concentration of the *H. perforatum* plant and it was observed to be at





	Calendula officinalis	Trachystemon orientalis	Hypericum perforatum
4%	80,45	80,38	90,38
8%	82,34	80,8	92
16%	85,5	89,26	85,03
20%	83,53	93,11	87,9

Fig. 1 — Microtiter plate biofilm test of *E. coli*. (A)The plates were painted with crystal violet. The EOs of inhibited biofilm formation at different levels. (B) Expressed as a percentage depending on the Formula 1. [All data are statistically significant (P <0.05)]

Table 1 — Anti-QS activity of the essential oils against *C. violaceum* ATCC 12472

Samples	Effective concentrations(w/v)	Zone of pigment inhibition (mm)
Calendula officinalis	4%	16,67±3,06
	8%	16,33±3,21
	16%	15,67±3,79
	20%	15±4,36
Hypericum perforatum	4%	9±7,94
	8%	13,33±2,31
	16%	14±2,64
	20%	14±2
Trachystemon orientalis	4%	16,67±4,16
	8%	19,33±4,04
	16%	19,33±1,15
	20%	19±3,61

[Values are given as mean ± standard deviation. All data are statistically significant (P <0.01)]

Table 2 — TAS, TOS and OSI values of plant essential oils

	TAS (mmol/L)	TOS (μmol/L)	OSI(TOS/ (TAS×10))
Calendula officinalis	4,72±0,15	8,60±0,74	0,182±0,009
Hypericum perforatum	5,36±0,12	10,06±0,73	0,188±0,009
Trachystemon orientalis	5,14±0,14	7,19±0,36	0,140±0,005

[TAS, Total antioxidant status; TOS, Total oxidant status; OSI, Oxidative stress ındex. Values are given as mean ± standard deviation. The tests were done in 3 repetitions]

9 mm levels. In Table 1, the inhibition zones of the study performed with 3 replications are given by calculating the standard deviations.

Antioxidant and oxidant capacity

In the study, antioxidant and oxidant levels of three plants EOs were determined with Rel Assay Diagnostic kits. As seen in Table 2, *C. officinalis* EO TAS value was 4.72 mmol/L, while TOS value was 8.60 μmol/L; *H. perforatum* EO TAS value was 5.36 mmol/L, TOS value is 10.06 μmol/L; and *T. orientalis* EO showed 5.14 mmol/L and 7.19 μmol/L, respectively. By proportioning these results according to the formula, the OSI values are found to be 0,182; 0,188; 0,140, respectively.

Discussion

Plant EOs have been used in alternative medicine for thousands of years as a natural antimicrobial and antiviral agent[2]. EOs consist of a complex composition and these properties enable them to have multiple targets in microbial cells. It changes the fluidity of the cell membrane by disrupting the lipid packaging in the microbial cell envelope through pathways such as cell content leakage and proton motive force inhibition and drives microorganisms to cellular lysis[22]. Treatments aimed at destroying bacteria create stress in bacteria and as a result cause them to develop resistance in different ways, especially biofilm[23]. It has been a remarkable approach recently to prevent bacteria from forming QS by interrupting the communication between them. The fact that EOs are natural QSI sources has increased studies in this area[22]. It is important to investigate the oxidant capacity as well as the antioxidant value of the plant. The OSI value shows to what extent the antioxidant compounds in the plant can suppress oxidant substances. The increase in the OSI value is explained by the insufficient antioxidant compounds in the plant and the intense oxidant substances[24].

The aim of this study is to evaluate the antioxidant and oxidant capacities of EOs obtained from three different plants, their role in interfering with the bacterial QS mechanism and whether they affect biofilm formation in *E. coli*, a selected bacterial pathogen. Two of the EOs we selected belong to the medicinally important plants *H. perforatum* and *C. officinalis*. *T. orientalis* is a plant that grows widely in Turkey, but detailed studies on its bioactivity are not sufficient. In the literature, no studies were found on the biofilm and anti-QS effects of plant EOs. This study shows that each EO can inhibit bacterial biofilm formation and impair QS signals at low concentrations.

In a study conducted in 2017, it was determined that *C. officinalis* extracts had a very high effect on removing and preventing biofilm formation against biofilm-forming bacteria isolated from infected eyes and contact lens cases[25]. In the study conducted by Tosun *et al*.[5] showed both methanol and ethanol extracts of *C. officinalis* plant antiquorum sensing effect against *Chromobacterium violaceum* 026 *and Agrobacterium tumefaciens* A136 biomonitor strains[5]. In various studies conducted with EO obtained from *C. officinalis*, it has been determined to have antibacterial and antifungal effects, and it has also been reported to have a high antioxidant capacity[26-28]. In current study, *C. officinalis* EO showed an inhibition effect of over 80% in biofilm formation at 4 different concentrations, while values above 15 mm determined by the well diffusion method revealed that the anti-QS effect was quite high. *C. officinalis*, which has a moderate antioxidant capacity (4.72 mmol/L), has a low OSI value due to its low oxidant accumulation (8.60 μmol/L). These results align with previous studies with total extracts and show that the EO of the plant is valuable from a pharmacological point of view.

While there are many studies in the field of antibacterial activity for another herb, *H. perforatum*, studies on its antibiofilm and anti-QS properties are few. In a study, the anti-QS effect of ethanol extracts of *H. perforatum* against MRSA was investigated by the δ-toxin production method and no significant inhibition was observed[29]. In another study conducted with ethanol, methanol, acetone and ultrasonication extracts, moderate inhibition effects were observed on two different QS signaling pathways, while none of the extracts prevented biofilm formation[3]. In a study conducted against *Staphylococcus aureus* with the ethanol extract of *H. perforatum*, a 56.85% reduction in biofilm formation was observed as a result of the microplate biofilm test. In the same study, biofilm formation was also measured quantitatively and *H. perforatum* ethanol extract impregnated on polyurethane discs reduced biofilm formation by 92.85%[30]. Although this result is in parallel with current study, a biofilm inhibition effect of *H. perforatum* EO against *E. coli* was observed at 8% concentration and 92%. In this study, *H. perforatum* EO showed moderate anti-QS effect depending on the concentration. It is possible that the compounds in EOs act individually or synergistically as inhibitors of QS. In addition, the highest TAS value among the EOs we studied was found in the *H. perforatum* plant with a value of 5.36 mmol/L. It is known that this plant, which has a very low OSI value (0.188 AU), is a medicinally valuable plant and is currently used in pharmacological studies.

No previous EO study of the *T. orientalis* plant has been found. In a study with methanol extract, it was reported that the phenolic and flavonoid compounds in the plant content increase the antioxidant value and have many biological activities such as antimicrobial and antitumor. The extract showed the highest antimicrobial effect against *E. coli* bacteria[31]. These data are compatible with current study. Plant EO has a high antioxidant value (5.14 mmol/L). While biofilm formation was inhibited by 93.11% depending on the concentration, the anti-QS effect was found above 19 mm. It is an expected result that this plant, which has a high antimicrobial effect, also has high antibiofilm and anti-QS effects.

Conclusion

In the conducted study, the Total antioxidant status (TAS) and Total oxidant status (TOS) levels of Essential oils (EOs) derived from *C. officinalis*, *H. perforatum* and *T. orientalis* were assessed, and their Oxidative stress ındex (OSI) values were calculated, revealing notably low levels. EOs exhibiting
low OSI levels are anticipated to possess heightened phytotherapeutic efficacy. The current findings meet this ancipation and demonstrate that EOs have robust anti-QS and antibiofilm effects. While each plant EO significantly inhibited *E. coli* biofilm formation (>80%), they also exhibited varied degrees of interference with the QS mechanism of the *C. violaceum* ATCC 12472 biosensor strain (≥9; <20). Notably, the variation in the efficacy of plant EOs observed within cellular environments underscores the need for further investigations, potentially at the molecular level or through *in vivo* studies. This research is an important basic for future studies aimed at understanding of the therapeutic potential of EOs.

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Conflict of Interest

Authors declare no competing interests.

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