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Anti-quorum sensing activity of olive leaf microwave-assisted extract

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Abstract
Quorum sensing (QS) controls the production of virulence factors in many pathogenic bacterial species, making QS inhibition a potential strategy for preventing antibiotic resistance. This study aimed to investigate the effect of different extraction methods including conventional solvent (CS), microwave-assisted (MA), and ultrasound-assisted (UA) extraction of olive leaf on QS inhibition. Olive leaf was extracted using methanol:water (MW) (50/50, v/v), ethanol:water (EW) (50/50, v/v), and water (W). The extracts were also analyzed for their total phenolic content, with the microwave-assisted water extract (MAW) showing the highest amount (113.33 ± 0.94 mg GAE/g), while the classical solvent water extract (CSW) had the lowest content (24.46 ± 0.97 mg GAE/g). The MAW demonstrated better antioxidant capacity with a values of 316.62 ± 7.82 µmol Trolox/g (DPPH method) and 314.45 ± 1.12 TEAC/mg (ABTS method). Moreover, CSEW, CSMW, UAEW, MAW, and MAEW extracts exhibited antibacterial activity against all test bacteria except *Escherichia coli* by agar well diffusion method. The extracts were also evaluated for their QS inhibition effects on model bacteria, including *Chromobacterium violaceum* (*C. violaceum*) CV026, *C. violaceum* CECT 494, and *Pseudomonas aeruginosa* PA01. The microwave-assisted water extract exhibited QS inhibition without affecting bacterial growth, as confirmed by qRT-PCR analysis at minimum inhibition concentration (MIC)/2 (1.875 mg/mL), MIC/4 (0.938 mg/mL), and MIC/8 (0.469 mg/mL). Additionally, the SEM images provided evidence of extract homogeneity. The present study demonstrates the potential of a microwave-assisted water extract of olive leaf, obtained through green technology and using a non-toxic solvent, as a natural antibacterial agent.

Keywords Microwave-assisted extraction · Olive leaf · Quorum sensing inhibition · Ultrasound-assisted extraction

1 Introduction

A large number of studies have been conducted over time to evaluate and utilize agricultural by-products that would otherwise be discarded. Among these by-products, the leaf of the evergreen olive tree (*Olea europaea* L.) have been the subject of considerable interest and study for centuries. Olive leaf extracts are a rich natural source of bioactive compounds, including oleuropein, tyrosol, and hydroxytyrosol. These compounds exhibit a range of biological activities, including antioxidant, antibacterial, and anti-quorum sensing properties [1].

The field of green chemistry, which seeks to recover phenolic compounds from plants, plays a significant role in the pursuit of sustainable development [2–4]. The concept of green chemistry entails the development of novel chemical reagents and reaction conditions in chemical synthesis, with the objective of promoting resource efficiency, energy efficiency, product selectivity, simplicity of operation, health and environmental safety [5–7]. The applications in question

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are utilized across a range of industrial sectors, including energy, chemicals, pharmaceuticals, food, waste recycling, and treatment [8]. Green extraction methods, such as microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), have been employed as effective, modern, and rapid approaches that reduce the use of organic solvents and increase extraction efficiency [9]. Furthermore, these extraction methods offer a substantial opportunity for the extraction of plants with antioxidant, anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, as well as beneficial cardiovascular effects [10].

The antimicrobial activities of a variety of plant species have been the focus of significant research in recent years. Nevertheless, the focus of the scientific community has now shifted towards identifying plant-derived compounds that can effectively regulate biofilms and virulence factors in pathogenic microorganisms, rather than acting upon them directly. In this specific context, there has been a concerted effort to isolate naturally occurring anti- quorum sensing (QS) agents, owing to their specificity and comparatively reduced toxicity [11, 12]. Several plant extracts, essential oils, and nanoparticle-based formulations [13] have been extensively studied for their efficacy in counteracting biofilm formation-related issues [14]. The biofilm formation is the underlying cause of the significant impact that pathogenic microorganisms have on the health and life of organisms. The impact of QS-regulated biofilm formation is significant in various food industries. It leads to many concerns, including food spoilage, disease outbreaks, biofilm-associated deaths, and a burden on the economy [15]. So, QS and biofilm formation are essential mechanisms that warrant further investigation to develop effective strategies for preventing microbial communication [16]. The QS has been demonstrated to regulate the pathogenicity or virulence, expression of genes important for biofilm formation, and regulation of various physiological activities in most pathogenic bacteria [17]. Moreover, a multi-layer defense system is formed within biofilms through the development of persister cells, the expression of adaptive stress responses, limited antibiotic penetration, restricted nutrition, reduced growth and metabolic activity, and the inactivation of antimicrobials within the extracellular polymeric substance matrix [18]. The QS systems in Gram-negative bacteria involve LuxI-type autoinducer synthesis, which produces specific acylated homoserine lactone (AHL) autoinducers. AHLs bind to the LuxR receptor protein, triggering specific gene expression. For *P. aeruginosa* PA01, a Gram-negative bacterium, it has been reported that three extensively studied interrelated and integrated QS systems, LasI/R, RhlI/R and PQS, are mutually regulating and self-regulating [19]. The QS system in *C. violaceum* includes the CviI/R system, a LuxI/R homolog system activated in response to the autoinducer *N*-hexanoyl-1-homoserine lactone (HSL) [20, 21]. Bacteria employ different types of QS signaling molecules to synchronize the expression of specific genes involved in biofilm formation and virulence. In *C. violaceum* CV026, violacein production is mediated by the *vio*ABDEC operon, which is regulated by the cviI/R system. The *vio*ABDEC operon consists of five enzyme-coding genes, mainly oxidoreductases and mono-oxygenases, including *vio*A, *vio*B, *vio*D, *vio*E, and *vio*C [22].

The objective of this study is to evaluate a comparative analysis of the various extraction methods employed to ascertain their impact on the total phenolic content of olive leaf. The antioxidant capacities, antibacterial activities, and antiquorum sensing properties of olive leaf extracts at the molecular level were also analyzed and compared with the culture method. To assess the potential effect of olive leaf extracts on violacein production, temporal variations in phenotypes and gene expression patterns were examined in relation to cell density. In this context, olive leaf were extracted using conventional solvent extraction, MAE, and UAE methods, and the antibacterial activities of the extracts and their effects on QS (bacterial communication/environmental sensing) were evaluated.

2 Materials and method

2.1 Chemicals

All chemicals used were of analytical grade and purchased from E. Merck (Darmstadt, Germany) and Sigma Chemical Company (Sigma-Aldrich GmbH, Steinheim, Germany). The chemicals used were as follows: gallic acid, ascorbic acid, Folin–Ciocalteu reagent, 1,3,5-tri(2-pyridyl)-2,4,6-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Muller Hinton Agar (MHA), Nutrient Agar (NA), Nutrient Broth (NB), Luria Bertani Agar (LBA), Luria Bertani Broth (LBB), Brain Heart Infusion (BHI) agar, *N*-Hexanoyl-L-homoserine lactone (C6HSL), and Dimethyl sulfoxide (DMSO). Hybrisol was obtained from Hibrigen Biotechnology R&D Industry and Trade Inc. (Kocaeli, Turkey) for DNA isolation from plant and microorganism tissues.

2.2 Plant materials

The plants used in the study were olive leaves collected during the first harvest period (October) from an olive orchard with Gemlik variety olive trees (Olea europaea) grown by a local farmer in Adana province in the Mediterranean Region of Turkey. The collected leaves were washed and dried at 40 °C until a constant moisture level of 6% was attained. The dried olive leaf were then ground using an electronic grinder (IKA-A-11, İstanbul, Turkey).

2.3 Microorganisms and culture conditions

Staphylococcus aureus (*S. aureus*) ATCC 29213, *Escherichia coli* (*E.coli*) ATCC 25922, *Bacillus subtilis* (*B. subtilis*) ATCC 11774, and *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 13883 were used in all experiments and obtained from the American Type Culture Collection (ATCC). These microorganisms were cultured twice on BHI (Brain Heart Infusion) (Scharlau Chemie S.A., Barcelona, Spain) agar plates for 24 h at 37 °C. In the evaluation of quorum sensing inhibition effect, *C. violaceum* CV026, *C. violaceum* CECT 494, and *Pseudomonas aeruginosa* PA01 were used as model microorganisms. *C. violaceum* CV026 is a mini-Tn5 mutant of the *C. violaceum* CECT 494 strain, whose violacein production depends on exogenous auto-inducer addition. The wild-type *C. violaceum* CECT 494 was obtained from the Spanish Type Culture Collection (Valencia, Spain). *P. aeruginosa* PA01 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ).

2.4 The process of extracting the olive leaf

Three grams of dried olive leaf were extracted with 50 mL of solvent. In the extraction processes methanol:water (50/50; v/v) (MW), ethanol:water (50/50; v/v) (EW), and distilled water (W) were used as solvents. After each extraction, the solvents used in the solutions were evaporated using a rotary evaporator (Buchi R-210, New Castle, USA). The obtained olive leaf extracts (OLE) were transferred to a 500 mL flask with a neck and lyophilized (CHRIST Alpha 1–4 LDplus, Osterode am Harz Germany) overnight at − 80 °C. The freeze-dried samples were prepared with water at appropriate concentrations and used in the analyses. Samples were prepared in duplicate for all solvents.

2.4.1 Classical solvent extraction (CSE)

The dried olive leaf were weighed at 3 g after grinding. The raw material and solvent mixtures were mixed with a magnetic stirrer (IKA C-MAG HS 10, İstanbul, Turkey) at 1500 rpm for 1 h. The mixtures were filtered using Whatman (42) filter paper to remove large particles. The evaporation was applied to remove the remaining solvents in the extracts prepared with ethanol and methanol using rotary evaporator (Buchi R-210, New Castle, United States) [23].

2.4.2 Ultrasound-assisted extraction (UAE)

3 g of dried and ground olive leaf were weighed into beakers for ultrasound-assisted extraction. Water, ethanol (50%; v/v), and methanol (50%; v/v) were used as solvents. The extraction was carried out using an ultrasonic device (Branson SFX 250, Mexico) with an output power of 250 W and an ultrasonic frequency of 20 kHz, containing an ultrasonic probe. The extraction condition was determined as 30% vibration amplitude and 5 min extraction time [24]. Sonication was applied in pulse mode, and the samples were kept in an ice bath during extraction for temperature control. Following the extraction process, the samples were subjected to centrifugation (Hettich-Rotofiks 32 A, Tuttlingen, Germany) at 5000 rpm for 15 min.

2.4.3 Microwave-assisted extraction (MAE)

3 g of dried and ground olive leaf were weighed into beakers for microwave-assisted extraction. MAE was applied to olive leaf samples prepared using water, ethanol (50%; v/v), and methanol (50%; v/v) using an Ethos-one (Milestone, Italy) device under 1 atm pressure at 30 °C with a 50% rotation speed for 5 min [25].

2.5 Total phenolic content

The extracts' total phenolic content (TPC) was assessed by the Folin-Ciocalteu assay with slight modifications [26]. Diluted extracts were taken, and 0.5 mL of 50% Folin-Ciocalteu reagent and 0.5 mL of 20% sodium carbonate were added. Absorbance values were determined at 765 nm using a UV–Vis spectrophotometer (Agilent Cary-60, Santa Clara, USA) after 30 min of incubation in the dark. TPC was expressed as mg/g gallic acid equivalents.

2.6 Determination of radical scavenging effects by DPPH· and ABTS•+ Scavenging methods

The antioxidant activity of olive leaf extracts was determined using the ABTS + radical, following the methodology described by Rufino et al. [27]. Samples were prepared at a concentration of 60 mg/mL. The absorbance value was measured at 734 nm. Trolox was used as an external standard for standardization, and the results were expressed as antioxidant activity in TEAC/g dried leaf. The DPPH• free radical scavenging assay [28] was used to determine the antioxidant activities of different OLE. The concentration of the DPPH• solution prepared in methanol was calculated spectrophotometrically at 515 nm and it was expressed in µmol Trolox/g dried leaf.

2.7 Antibacterial activity

2.7.1 Agar well diffusion assay

The antibacterial activities of the extracts were determined using the agar well diffusion method. *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *B. subtilis* ATCC 11774, and *K. pneumonia* ATCC 13883 were used as test microorganisms. Bacteria were incubated in tryptic soy agar for 24 h at 37 °C. Certain amounts of activated pure culture were transferred into sterile 0.9% NaCl solution and adjusted according to McFarland 0.5 (10^8 CFU/mL) to form standard turbidity. 100 µL of this bacterial solution was taken and spread on MHA medium using the spread plate method. The prepared extracts were added at 80 µL amount into the wells on the medium with well diameter of 7–8 mm. Chloramphenicol was used as the positive control and sterile water was used as the negative control in the assay. Petri dishes were incubated at 37 °C for 24 h. At the end of the incubation period, the inhibition zones around the wells were measured with a caliper. Two parallel petri dishes were prepared, and 3 measurements were taken from each zone [29].

2.7.2 Minimum inhibitory concentration (MIC)

MIC values of the extracts were determined according to agar well-diffusion method. *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *B. subtilis* ATCC 11774, and *K. pneumonia* ATCC 13883 were used as test organisms. The activated pure cultures were adjusted to a McFarland standard 0.5 (10^8 CFU/mL). Subsequently, 100 µL of the bacterial solution was spread on an MHA medium. The samples were then diluted in two-fold dilutions (1.87–60 mg/mL) and added to the wells. The test was conducted in duplicate. Petri dishes were incubated at 35 °C for 18–24 h. Results are given as zone diameter (mm) ± standard deviation.

2.8 Anti-quorum sensing (anti-QS)

2.8.1 Qualitative screening for QS inhibition by OLE

To determine the anti-QS activity of OLE, a bioreporter strain, *C. violaceum* CV026, was employed. The LB agar plates were prepared by adding hexanoyl homoserine lactone at 0.125 µg/mL and antibiotic kanamycin at 20 µg/mL. For MIC

analysis, a suspension of 100 µL of an overnight-grown culture of *C. violaceum* was spread on an agar surface. 80 µL of two-fold dilution concentrations (1.87–60 mg/mL) of OLE was added to the wells, and the plates were incubated at 30 °C overnight. The formation of colorless colonies was then observed.

2.8.2 Quantification of violacein production in *C. violaceum*

The assay was performed as described by Tan et al. [30] with modifications. *C. violaceum* CV026 was cultivated at 30 °C, and the assay was performed in test tubes. The positive control for inhibition of quorum sensing was prepared with the addition of 5 mL of melted LB agar (1% w/v) to which 50 µL of autoinducer C6HSL and 100 µL of a culture of *C. violaceum* CV026 had been previously added, and subsequently inoculated. Additionally, The quantitative estimation of the violacein pigment was conducted utilizing the strain *C. violaceum* CECT 494, by the methodological approach proposed by D'Almeida et al. [31]. *C. violaceum* CECT 494 culture was grown in LB broth, and the extract was added at a concentration of 60 mg/mL. A culture medium without extract was prepared as the negative control. After incubation, the culture solution was subjected to centrifugation at 5000×*g* for 10 min. The pellet was then dissolved using dimethyl sulfoxide and the absorbance at 585 nm was determined. The test was carried out in triplicate, and the percentage inhibition of violacein production was calculated following Eq. (1):

$$\%\ \text{inhibition of violacein production} = \frac{\text{OD of control at 585 nm} - \text{OD of treatment at 585 nm}}{\text{OD of control at 585 nm}} \times 100 \quad (1)$$

2.8.3 Swarming motility assay

In brief, the agar plates with LB medium were prepared for swarming agar plates, and the pH was kept at 7.0. The *P. aeruginosa* PA01 culture was subjected to an overnight treatment in the presence of OLE. The 10 µL aliquot of the overnight culture was dispensed onto the center of a swarm plate and incubated at 37 °C overnight. Thereafter, observations of swarming migrations were undertaken, with the measurement of their zones (mm) [32].

2.9 Quantitative real-time PCR (qRT-PCR)

RNA samples were extracted using Hybrisol (Hibrigen Biotechnology R&D Industry and Trade Inc., Kocaeli, Turkey) following the instructions. The cDNA was prepared using the Onescript cDNA Synthesis kit (abm, Cat no. G236, Canada). The PCR mixture was prepared at a 10 µL volume, comprising 1.0 µL of cDNA sample, 5 µL of 2 × SYBR Green Master Mix, 0.2 µL of forward primer F and reverse primer R, and 3.6 µL of DNase-Free water. The data regarding the gene expression of the *C. violaceum* CV026 QS system was collected using the qRT-PCR instrument, the Rotor-Gene Q, produced by Qiagen in Hilden, Germany. PCR was performed at 94 °C for 2 min, followed by 45 cycles (59 °C for 30 s, 30 s at the specific primer annealing temperature, and 72 °C for 30 s). The primers used for qRT-PCR were described in Table 1 [33]. The specificity

Table 1 The primers used for qRT-PCR

Gene	Primers	Nucleotide sequence (5′–3′)	Annealing temperature (°C)
16 S	Forward	GCGCAACCCTTGTCCTTAGTT	59
	Reverse	TGTCACCGGCAGTCTCCTTAG	59
cvil	Forward	CTGAAACTAAGCTGCGACAGTTG	59
	Reverse	GAAACCGTCCTCGCATAAGG	59
cviR	Forward	CCCAGCAATATGCCGTATC	59
	Reverse	CATTGAGCTTGCGGATCACA	59
vioB	Forward	TGGAACAGGAAGTGCGGATG	59
	Reverse	CCGGCAACAACCATTTCTCC	59
vioC	Forward	GATCATATCGCCCTGCAAGC	59
	Reverse	CCGCTACTACTTCGAGCACA	59
vioD	Forward	GTACTCGGACACGATGAGCAC	59
	Reverse	CACCTTGGCGACGTATTCGG	59

of the qRT-PCR results was determined by melting curve analysis of the amplified products using the standard method. All reactions were conducted in triplicate, and the relative expression of genes ($2^{-\Delta\Delta Ct}$) was calculated using Eqs. (2 and 3) [34], where Ct is the cycle threshold of samples:

$$\Delta Ct = Ct\,(\text{gene of interest}) - Ct\,(\text{housekeeping gene}) \quad (2)$$

$$\Delta\Delta Ct = \Delta Ct\,(\text{treated sample}) - \Delta Ct\,(\text{untreated sample}) \quad (3)$$

2.10 Scanning electron microscopy analysis (SEM)

The morphology of OLE samples extracted using different methods was evaluated using a scanning electron microscope (SEM) system (FEI, Quanta 650 Field Emission SEM) operating at an accelerating voltage of 15 kV. A thin layer of gold was sputtered onto the samples using a magnetron sputtering machine (Quorum, Q150R ES, England) under vacuum conditions, and images were recorded at 250 × magnification.

2.11 Statistical analysis

Each analysis was done in triplicate, and the resulting data were expressed as the mean ± standard deviation (n = 3). Multiple comparisons of means (one-way ANOVA with Fisher pairwise comparison test) were used to carry out comparisons between groups. Significance was tested at $p < 0.05$.

2.12 Ethical approval

The collection of the plants used in the study complies with national guidelines with no need for further affirmation.

3 Results and discussion

3.1 Total phenolic content and antioxidant capacity

The total phenolic content of the extracts ranged from 24.46 to 113.33 mg GAE/g for different extraction methods (Table 2). The highest total phenolic content was determined for the microwave-assisted extract using water as the solvent

Table 2 The extracts' total phenolic content (TPC) and antioxidant activity (using DPHH and ABTS methods)

Samples	TPC (mg GAE/g)	DPPH (µmol Trolox/g)	ABTS (TEAC/mg)
CSMW	31.83 ± 0.77^{f}	195.11 ± 4.69^{e}	316.52 ± 2.40^{c}
CSEW	38.36 ± 1.54^{d}	220.67 ± 5.49^{d}	215.12 ± 2.60^{f}
CSW	24.46 ± 0.97^{g}	104.65 ± 3.86^{g}	310.25 ± 4.61^{d}
UAMW	35.54 ± 5.24^{e}	130.80 ± 6.36^{f}	314.45 ± 2.26^{cd}
UAEW	39.68 ± 0.68^{d}	258.24 ± 9.89^{b}	111.08 ± 3.01^{g}
UAW	26.83 ± 0.22^{g}	114.19 ± 2.59^{g}	54.05 ± 1.28^{h}
MAMW	81.95 ± 0.56^{c}	250.93 ± 11.03^{bc}	459.22 ± 3.64^{b}
MAEW	88.17 ± 0.30^{b}	241.04 ± 6.91^{c}	278.41 ± 2.19^{e}
MAW	113.33 ± 0.94^{a}	316.62 ± 11.59^{a}	503.72 ± 2.38^{a}

CSMW Classical solvent methanol:water extract, *CSEW* Classical solvent ethanol:water extract, *CSW* Classical solvent water extract, *UAMW* Ultrasound assisted methanol:water extract, *UAEW* Ultrasound assisted ethanol:water extract, *UAW* Ultrasound assisted water extract, *MAMW* Microwave-assisted methanol:water extract, *MAEW* Microwave-assisted ethanol:water extract, *MAW* Microwave-assisted water extract

Data are means ± SD (n = 3)

[a–g]In each column, means with different letters show statistically significant differences (one-way ANOVA Fisher pairwise comparison test, $p < 0.05$)

(MAW). The antioxidant capacity analysis results were correlated with the total phenolic content, showing the highest antioxidant capacity in the MAW. The results demonstrated a statistically significant difference between the samples at the $p < 0.05$ level. The antioxidant capacity of this extract was 316.62 ± 7.82 µmol Trolox/g with the DPPH method and 503.72 ± 2.38 TEAC/mg with the ABTS method. This is believed to be due to the homogeneous heat conduction throughout the sample and higher molecular rotation in the MAE method. Moreover, the differences in solvent polarity influenced the extraction efficiency of different antioxidant compounds, affecting DPPH and ABTS results. The ABTS assay provided more consistent results across all solvent systems, while DPPH was more effective in ethanol/methanol-based extracts due to its lower solubility in water. The choice of solvent should have a high dielectric constant and effectively absorb microwave energy [35]. Several studies have investigated the antioxidant effects of phenolic compounds obtained from OLE. The extraction methods employed have demonstrated that the MAE method yields the highest phenolic content. These findings are in line with the results reported by Dobrincic et al. [36]. In a study comparing UAE and MAE techniques to determine the polyphenol and flavonoid content of *Olea europaea* leaf, MAE water extracts were found to have the highest content and were considered a fast, convenient, efficient, and environmentally friendly method [37]. In a previous study, raw olive leaf juice obtained through hydraulic pressing was administered to experimental mice as a source of polyphenols. The antioxidant activity of samples containing olive leaf juice was found to be higher than that of samples containing synthetic antioxidants [38].

3.2 Antibacterial activity and MIC values

The antibacterial activities of OLE against test microorganisms were determined using the well diffusion method, as shown in Table 3. Upon examination of the antibacterial activity results, it was determined that the extracts obtained through classical solvent extraction with ethanol–water (1:1) (CSEW) and methanol–water (1:1) solvents (CSMW), as well as the samples extracted with ethanol–water (1:1) (UAEW) through ultrasound-assisted extraction and the samples extracted with water (MAW) and ethanol–water (1:1) (MAEW) through microwave-assisted extraction, exhibited antibacterial activity. None of the nine extracts showed antibacterial activity against *E. coli* ATCC 25922.

MIC values of extracts (CSEW, CSMW, UAEW, MAW, and MAEW) showing antibacterial activity against the test microorganisms were determined at concentrations of 60, 30, 15, 7.5, 3.75, and 1.87 mg/mL. The MIC value for *K. pneumoniae* bacteria of the CSEW sample was found to be 7.5 mg/mL and also, it was found as 30 mg/mL for *S.aureus* of the CSMW sample. The MIC value of the remaining extracts for *B. subtilis* ATCC 11774, *K. pneumonia* ATCC 13883, and *S. aureus* ATCC 29213 bacteria was calculated as 3.75 mg/mL.

A number of studies have documented the antibacterial activity of dry OLE against a range of microbial organisms. Gutierrez et al. [39] observed comparable outcomes to our study with MA extracts. For instance, the MIC values of MAEW extracts against *S. aureus* CECT 5193 bacteria were lower (concentration of 20 mg/mL) than what was observed in this study, while the MIC value of MAW extract was higher than this study with a concentration of 2.5 mg/L. Giacometti et al. [40] reported the inhibitory effect of OLE obtained by ultrasound-assisted extraction and classical solvent extraction methods on the *E. coli* 25922 bacterial strain at a concentration of 8 mg/mL.

Table 3 Antibacterial activity and minumum inhibition concentration (MIC) of olive leaf extract (OLE)

Microorganisms	CSEW	CSMW	UAEW	MAW	MAEW
Inhibition zone (mm ± SD)					
B. subtilis ATCC 11774	11.14 ± 0.16	naa[a]	8.83 ± 0.25	10.40 ± 0.38	9.12 ± 0.52
K. pneumoniae ATCC 13883	13.26 ± 0.75	naa[a]	9.89 ± 0.66	10.32 ± 0.19	10.47 ± 0.34
S. aureus ATCC 29213	9.13 ± 0.07	12.93 ± 0.65	10.37 ± 0.24	10.37 ± 0.39	9.51 ± 0.29
Concentration (mg/mL)					
B. subtilis ATCC 11774	3.75	naa[a]	3.75	3.75	3.75
K. pneumoniae ATCC 13883	7.5	naa[a]	3.75	3.75	3.75
S. aureus ATCC 29213	3.75	30	3.75	3.75	3.75

CSMW Classical solvent methanol:water extract, *CSEW* Classical solvent ethanol:water extract, UAEW: Ultrasound assisted ethanol:water extract; extract, *MAEW* Microwave-assisted ethanol:water extract, *MAW* Microwave-assisted water extract

[a]naa: no antibacterial activity

Table 4 Anti-QS activity of OLE against *C. violaceum* CV026 and *C. violaceum* CECT 494

Samples	Inhibition zone (mm ± SD)	
	C. violaceum CV026	*C. violaceum* CECT 494
MAW	14.83 ± 0.39	13.60 ± 0.30
MAEW	16.77 ± 0.61	11.82 ± 0.80
MAMW	17.07 ± 0.26	12.72 ± 0.62
CSW	naa[a]	9.92 ± 0.21

MAW Microwave-assisted water extract, *MAEW* Microwave-assisted ethanol:water extract, *MAMW* Microwave-assisted methanol:water extract, *CSW* Classical solvent water extract

[a]naa: no antibacterial activity

Table 5 Minumum inhibition concentration (MIC) of the OLE against *C. violaceum* CV026 and *C. violaceum* CECT 494

Concentration (mg/mL)	MAW	MAEW	MAMW
Inhibition zone diameter (mm)			
C. violaceum CV026			
60	13.10 ± 0.40	15.83 ± 0.80	16.25 ± 0.36
30	11.44 ± 0.33	12.00 ± 0.14	12.31 ± 0.41
15	10.84 ± 0.08	10.49 ± 0.24	10.42 ± 0.25
7.5	9.51 ± 0.29	9.61 ± 0.17	9.23 ± 0.12
3.75	naa[a]	naa[a]	naa[a]
C. violaceum CECT 494			
60	13.35 ± 0.27	12.27 ± 0.43	12.97 ± 0.72
30	11.69 ± 0.33	11.19 ± 0.38	11.51 ± 0.30
15	10.64 ± 0.35	9.45 ± 0.26	9.99 ± 0.37
7.5	9.72 ± 0.42	naa[a]	naa[a]
3.75	naa[a]	naa[a]	naa[a]

MAW Microwave-assisted water extract, *MAEW* Microwave-assisted ethanol:water extract, *MAMW* Microwave-assisted methanol:water extract

[a]naa: no antibacterial activity

3.3 Inhibition of violacein production

The inhibition of violacein production in *C. violaceum* CV026 and *C. violaceum* CECT 494 strains was investigated by comparing extracts with the control. As shown in Table 4, samples MAW, MAEW, and MAMW significantly inhibited ($p < 0.05$) violacein production in both *C. violaceum* CV026 and *C. violaceum* CECT 494. In general, extracts obtained through the MAE method, which had high total phenolic content, exhibited stronger inhibitory effects. For example, at a concentration of 113.33 ± 0.94 mg GAE/g, the extract demonstrated better inhibition than the positive control used in the test. In cultures using CSW, *C. violaceum* CV026 suppressed bacterial growth. However, the suppression of *C. violaceum* CECT 494 bacterial growth and violacein production by this extract was not statistically significant.

Therefore, MIC values of the active extracts obtained by the microwave method using different solvents was examined through the agar well diffusion method. The zone diameters of the MIC values are shown in Table 5. The MIC value for *C. violaceum* CV026 bacteria of the MAW, MAEW, and MAMW samples were found to be 7.5 mg/mL and also, it was found as 15 mg/mL for *C. violaceum* CECT 494 of the MAEW, and MAMW samples. The MIC value of the MAW for *C. violaceum* CECT 494 was determined as 7.5 mg/mL. The effect of these three extracts on *C. violaceum* CV026 bacteria is illustrated in Fig. 1. Consequently, the opaque zone diameters increased with increasing concentrations in the petri dishes to which these *C. violaceum* CV026 extracts were added.

The measurement of violacein production by UV–Vis spectrophotometry revealed 83.49% inhibition by MAW at 60 mg/mL concentration, and MIC was 3.75 mg/mL with 28.35% inhibition. The extract with a concentration of 3.75 mg/mL MIC in methanol: water (50:50; v/v) showed the lowest inhibition. A study demonstrated that peppermint extract inhibited violasin production by *C. violaceum* 026 bacteria. As the concentration of mint extract decreased, the

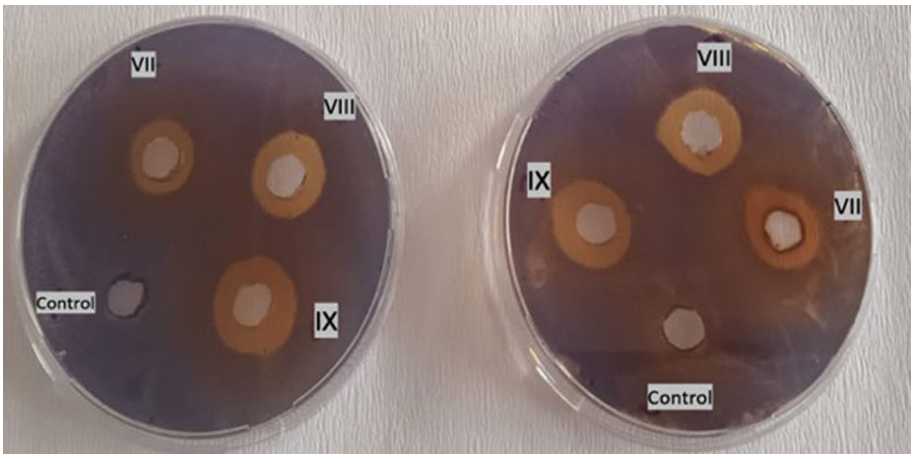


Fig. 1 Minumum inhibition concentration (MIC) of olive leaf extract (OLE) against *C. violaceum* CV026 using agar well diffusion method (control: C_6HSL, VII: MAW: Microwave-assisted water extract, VIII: MAEW: Microwave-assisted ethanol:water extract, IX: Microwave-assisted methanol:water extract

inhibition of violacin production also decreased. At a concentration of 400 µg/mL, the removal rate of violasin was 85%, while at 50 µg/mL, it was 26% [41]. A study by Vasavi et al. [42] reported that *Syzygium cumini* L. and *Pimenta dioica* L. extracts inhibited violacein pigment production in a concentration-dependent manner without inhibiting AHL synthesis, similar to our findings.

3.4 Quantification of swarming motility

The inhibition of swarming motility was evaluated using CSE and MAE in *P. aeruginosa* PA01, and the results are presented in Table 6. The OLE exhibited swarming motility inhibition ranging from 45.98% to 21.95%. Classical solvent extracts showed higher rates of swarming inhibition activity compared to MAE (Table 6). Ultrasound-assisted extracts had no effect on the QS inhibition of *P. aeruginosa* PA01 bacteria.

The growth inhibitory effect of the extracts on *P. aeruginosa* PA01 bacteria in the medium is illustrated in Fig. 2. Bacterial growth is indicated by a white/cream color, while the opaque areas represent non-growth regions. Notably, *P. aeruginosa* PA01 did not form branches at the edge of the breeding site but exhibited micro-sized tendrils, parallel with the findings presented by Morris et al. [43]. The zone diameter of the control region was larger than the zone diameter to which the CS and MA extracts were added. While not all QS organisms exert motility effects on *P. aeruginosa* PA01, it is important to consider that this effect may vary under different growth conditions and is dependent on cell density and condition.

Inhibiting biofilm formation maintains pathogens within a planktonic state, rendering them more susceptible to immune response and antibacterial agents [44]. The term "swimming motility" is used to describe the ability of individual cells to move through liquid or semi-solid media using flagella. In contrast, the term "swarming" is used to describe a coordinated, quorum-sensing (QS) related behaviour that enables groups of cells to move on semi-solid surfaces [45]. Therefore, it is necessary to investigate whether the extracts affect the motility of *P. aeruginosa* PA01 independently of the bacterial communication inhibition system. The inhibition effect of *P. aeruginosa* PA01 on swarming motility found in this study suggests that the extracts can directly affect motility and inhibit the QS system.

Table 6 Results of swarming effect of OLE on *P. aeruginosa* PA01

Samples	Swarming (%)
CSW	45.98 ± 1.82
CSEW	21.95 ± 0.99
CSMW	30.48 ± 0.97
MAW	41.37 ± 1.02
MAEW	28.33 ± 1.22
MAMW	42.30 ± 1.83

CSW Classical solvent water extract, *CSEW* Classical solvent ethanol:water extract, *CSMW* Classical solvent methanol:water extract, *MAW* Microwave-assisted water extract, *MAEW* Microwave-assisted ethanol:water extract, *MAMW* Microwave-assisted methanol:water extract

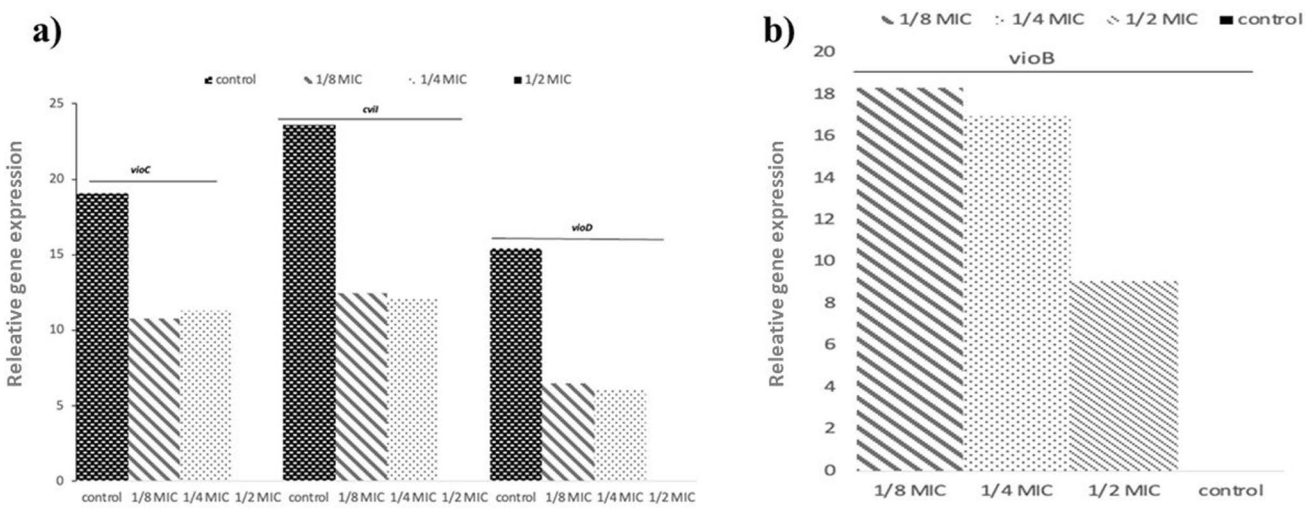


Fig. 2 **a** The suppressive effect of microwave-assisted extraction (MAE) extracts on genes under the control of the *CviI/CviR* system (Control: no extract was added) **b** Control: MIC value (3.75 mg/mL of OLE)

3.5 Quantitative real time-PCR (qRT-PCR)

To assess the inhibitory effect of quorum sensing at the molecular level, we investigated the efficiency of the extracts in regulating the expression of genes in *C. violaceum* CV026 bacteria using the Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) method. A qPCR calibration curve was generated for each primer set using cDNA from *C. violaceum* CV026 bacteria. Two primers, forward (F) and reverse (R), were used for amplification and sequencing of each gene target, while the small subunit 16S ribosomal RNA, served as the internal reference gene. The binding specificity of these primers was confirmed by generating a melting curve for each primer set, which exhibited a single sharp peak, eliminating the possibility of non-specific binding.

The results showed that the microwave-assisted water extract inhibited *cviI* and *cviR* gene expression at sub-MIC concentrations (consistent with other findings throughout the study) (Fig. 2). Controls using bacterial culture RNAs (without extract) were employed for *vioC, vioD,* and *cviI* genes. The extract with added MIC value was used to determine the inhibition of the *vioB* gene. The influence of the extracts on the expression of *vioB, vioC, vioD,* and *cviI* genes was observed at 1/2 (1.875 mg/mL), 1/4 (0.938 mg/mL), and 1/8 (0.469 mg/mL) MIC concentrations. The microwave-assisted water extract inhibited the expression of all four genes at all tested sub-MIC concentrations. However, not all concentrations exhibited an inhibitory effect on the expression of *vioA* and *cviR* genes. It has been reported that phenolic compounds can selectively kill or inhibit Gram-negative (–) and Gram-positive (+) bacterial cells. Additionally, a study examining the QS inhibition properties of various plant extracts found that OLE showed the strongest effect [46].

The results indicate that microwave-assisted water extract did not exhibit an inhibitory effect on *vioA* and *vioE* genes in *C. violaceum* CV026 at any concentration tested. It has been reported that these genes, are less critical during violacin biosynthesis when considering a statistical approach that combines 12 violacein pathways with individual gene components (*vioA, vioB*, *vioC, vioD*, and *vioE*) driven by a promoter [47]. At the upper threshold of cell population density in the CviI/CviR system, the constitutive gene CviI leads to the accumulation of C6-HSL. Our RT-qPCR results demonstrate that the microwave-assisted water extract inhibited the expressed genes encoding the CviI/CviR system and genes related to violacein biosynthesis.

3.6 Scanning electron microscopy analysis (SEM)

Figure 3 shows the SEM analysis images of methanol: water lyophilized extracts obtained using OLE with CS, UA, and MA methods. The observations made through SEM indicate that the extraction methods employed impacted the structure of the obtained extracts. The surface of the CSMW extract appeared rough, while the UAMW specimen

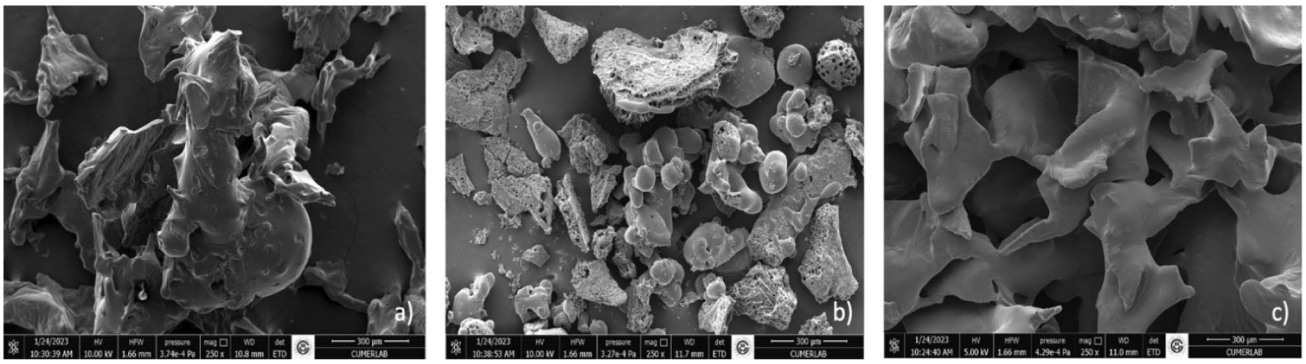


Fig. 3 Scanning Electron Microscopy (SEM) micrographs of extract powders (250×), **a** Classical solvent methanol:water extract (CSMW), **b** Ultrasound-assisted merhanol:water extract (UAMW), **c** Microwave-assisted methanol:water extract (MAMW)

exhibited a globular separation with small holes on its surface. The MAMW sample had a relatively smooth surface with a homogeneous appearance. The images showed consistent shape and structure among the samples obtained using MAE. The microwave-assisted extraction method enables rapid and homogeneous extraction of natural compounds from plant matrices by selectively heating polar molecules with microwave energy [48]. A study investigating SEM graphs of olive leaf after different extractions reported similar findings, where the extract powders exhibited a broken, rough texture, similar to our research. They suggested that the use of solvents caused fractures when oleuropein exited the cells by penetrating through the plant walls. Additionally, they concluded that ultrasonic waves induced cavitation and facilitated fracturing [49].

4 Conclusions

The properties of OLE were influenced by the extraction method (UAE, MAE, and classical solvent) and the solvent type (water, ethanol:water (50:50 v/v), methanol:water (50:50 v/v)). The combination of these factors affected the total phenolic content, antioxidant activity, antibacterial activity, and bacterial communication properties. Considering all the obtained results, MAE showed advantages over UAE and the classical solvent method, as it enabled successful extraction of olive leaf polyphenols with less solvent and in a shorter time. However, since the activity on microbial growth differs depending on the type of polyphenol, further investigation is necessary to determine the appropriate combination of polyphenols. Overall, microwave-assisted water extract of olive leaf demonstrated the ability to inhibit QS-regulated violacein pigment production in bacteria without impeding their growth, suggesting the potential of olive leaf as an antibacterial agent. However, more research is needed to elucidate its molecular mechanisms.

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Data availability Data is provided within the manuscript or supplementary information files.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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