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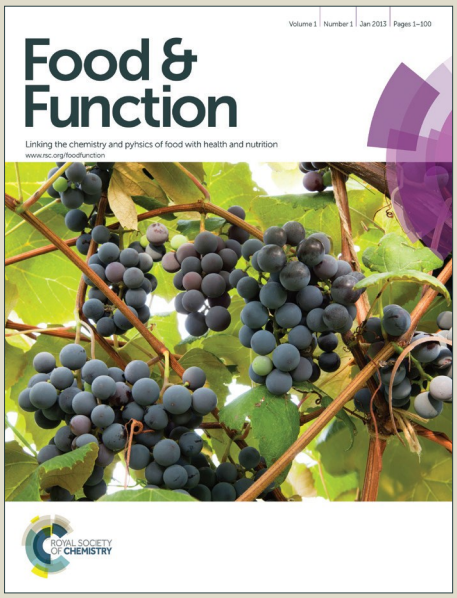

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Heracleum orphanidis: chemical characterisation, comparative evaluation of antioxidant and antimicrobial activity with specific interest in the influence on *Pseudomonas aeruginosa* PAO1

Running title: *Heracleum orphanidis* chemical composition and bioactivities

Ksenija S. Mileski[1], Ana D. Ćirić[2], Snežana S. Trifunović[3], Mihailo S. Ristić[4], Marina D. Soković[2],Vlado S. Matevski[5, 6], Vele V. Tešević[3], Milka B. Jadranin[7], Petar D. Marin[1], Ana M. Džamić[1,*]

[1]Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia
[2]Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia
[3]Faculty of Chemistry, University of Belgrade, Serbia
[4]Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia
[5]Faculty of Natural Sciences and Mathematics, University "S. Kiril and Metodij", Skopje, Macedonia
[6]Macedonian Academy of Sciences and Arts, Skopje, Macedonia
[7]Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia

*Corresponding author. (Dr. Ana Džamić: Tel.:+381113244498; Fax:+381113243603;*E–mail adress*: simicana@bio.bg.ac.rs)

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Abstract

GC–FID and GC–MS were used to determine the chemical composition of essential oil of aerial parts of *Heracleum orphanidis* Boiss. Also, HPLC–DAD/ESI–ToF–MS profile of methanol extracts of aerial parts and roots was determinated. The main components of the essential oil were *n*–octanol, octyl hexanoate and *n*–octyl acetate, while coumarins were the most prevalent compounds in methanol extracts. An evaluation of antioxidant activity showed that methanol and aqueous extract of aerial parts had the highest potential. In terms of antimicrobial activity determined by microdilution assays, essential oil and methanol extract of roots showed the greatest effectiveness. The colonies of *Pseudomonas aeruginosa* PAO1 treated with *H. orphanidis* samples produced less toxic pyocianin, showed lower twitching and flagella mobility and biofilm formatting was reduced. The analyses in this study showed considerable biological potential of *H. orphanidis* considering free radicals and various pathogenic strains, including wild type of *P. aeruginosa*.

Keywords: *Heracleum orphanidis*; Essential Oil; Antioxidant Activity; Antimicrobial Activity; Anti–quorum Sensing Activity, *Pseudomonas aeruginosa*

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1. Introduction

The genus *Heracleum* L. consists of about 70 species widely distributed in the northern hemisphere[1]. Species of this genus are characterized by simple, pinnate or 2–pinnate leaves and white, greenish–yellow or pink flowers[2]. In Macedonia, *Heracleum* is represented by two species – *H. orphanidis* Boiss. and *H. sphondylium* L. *H. orphanidis* is a biennial or perennial plant, up to 50 cm high, with unbranched or poorly branched steam and compose umbells with white petals. This species is endemic to the Balkan Peninsula[3].

It has been reported that roots, fruits and leaves of this genus are traditionally used as antipyretics, analgesics, digestives, diaphoretics and for their carminative and antiseptic properties[4,5]. Many *Heracleum* species are edible and medicinal plants, such as *H. persicum* which is extensively used as flavoring agent and spice for food in many parts of Iran. Firuzi *et al.*[6] reported its fruits as digestive and analgesic spice in soups and stews, while young stems are used for making pickles[7]. American species *H. maximum,* which is usefull against respiratory ailments including tuberculosis, was part indigenous people diet[8]. *H. dissectum* young stems and leaves are used as delicious vegetables and natural antihypertensive remedy by local people in China[9], while *H. siamicum* fruits as spices and as a carminative herbal drug in Thailand[10]. Tea made from *H. platytaenium* leaves and fruits are helpful as folkloric drug for gastritis, enteritis and in the treatment of the epilepsy [11]. *H. rigens* is beneficial in curing constipation, stomachache, diarrhea, gastric disorders and indigestion[12]. In France, *H. sphondylium* is used for a liqueur preparation, while in some Asian countries as food or food additive[13]. The roots of *H. sphondylium* and *H. nepalense* are known to regulate menstrual problems, high blood pressure, dyspepsia, digestive problems and diarrhea[14,15].

The chemical composition of essential oils (EO) of various *Heracleum* species (*e.g. H. sprengelianum*, *H. persicum*, *H. platytaenium*, *H. crenatifolium*, *H. sphondylium, H. anisactis*) hane been previously reported. The major groups of compounds found in *Heracleum* EOs mostly were aliphatic esters and alcohols, so as terpenoids[13,16–19]. Literature survay on chemical characterisation of extracts (Es) of *Heracleum* species revealed furanocoumarins, furocoumarin dimers, coumarin glycosides, anthraquinones, stilbene and alkaloids as their main bioactive constituents[20–23]. Coumarins and furocoumarins as the most frequent compounds of the genus are known for wide spectrum of pharmacological activities such as antioxidant, antimicrobial, antiviral, anti–inflammatory, cytotoxic, *etc*. Furanocoumarins are widely used in pharmaceutical

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industry as effective dermal photosensitizing agents, since they have potent stimulatory effect on melanogenesis with significant enhancement of cell proliferation. They are important drugs in vitiligo, psoriasis and leukoderma therapy of human skin[23,24–27].

Medical importance of *Heracleum* genus is confirmed by numerous studies on its biological activities. Based on available data, wide biological potential of *H. persicum* was found[28–31]. Hajhashemi *et al*.[26] reported antinociceptive and anti–inflammatory effects in the reduction of the pain and inflammation of *H. persicum*, supporting its traditional use. Immunomodulatory, anticonvulsant and strong citotoxic activities of different *H. persicum* extracts were also confirmed[28–30]. Besides, this species can be considered as potential glutathione–S–transferase inhibitor[31]. Moderate significance of antioxidant, antimicrobial, antitumor and cytotoxic activities of *H. persicum, H. transcaucasicum*, *H. pastinacifolium* and *H. rechingeri* was demonstrated by Firuzi *et al.*[6]. Dash *et al.*[15,32] reported significant *in vitro* and *in vivo* antioxidant, antimicrobial and stimulation of immune function activity of *H. nepalense* Es. Contrary, Es of *H. sphondylium* and *H. rechingeri* showed no remarkable antimicrobial potential[13,14,33].

Lately, discovering of new, sutible, antimicrobial agens is rapidly increasing since different pathogens have developed resistance toward syntetic antibiotics and antimycotics. It is known that pathogenic bacillum *Pseudomonas aeruginosa* causes nosocomial infections, bronchopneumonia, septic shock and wound infections. This bacterium employs quorum sensing (QS) mechanism to regulate its virulence and pathogenicity such as biofilm formation, swarming motility, pigmentation, production of pathogenicity factors, toxins, degradative enzymes and virulence factors. QS signal molecules are the main participants of intercellular signaling pathway in which bacteria communicate and regulate gene expression by releasing small compounds called autoinducers into the environment when they reach a certain population density. Due to its role in regulatory processes and survival of bacteria, inhibition of QS systems by disrupting or interfering within these communications between bacterial cells, is an important target for developing novel anti–infective agents[34–37]. Some publications reported anti–QS activity of certain medicinal plants[38–41].

The aim of this study was to characterize chemical composition of EO and methanol Es of *H. orphanidis*. According to the literature review, the chemical investigation of *H. orphanidis* Es has not been performed previously, thus a tentative analyzes of methanol Es reported in our

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study gives initial information about chemical composition of this plant. The final goal was to explore *H. orphanidis* for its *in vitro* antioxidant, antimicrobial and anti–QS effects using *P. aeruginosa* PAO1 as a model system. In addition, phenolic and flavonoid contents of Es were determined.

2. Materials and methods

2.1. Solvents and chemical reagents

For performing the experiments, solvents that were provided were of analytical grade. Most of organic solvents were procured from Zorka pharma, Šabac, Serbia. Acetonitrile (HPLC grade), purchased from Merck KG (Darmstadt, Germany), formic acid (85% pure), purchased from Lach–Ner, s.r.o. (Neratovice, Czech Republic) and Milli Q water 18.2 MΩcm, obtained from a Millipore Simplicity 185 purification system, were used for the LC–MS analyses. Gallic acid (98% pure), 3–tert–butyl–4–hydroxyanisole (BHA) (≥ 99%), 2,2–dyphenyl–1–picrylhydrazyl (DPPH) (≥ 99%), Folin–Ciocalteu phenol reagent, potassium acetate ($C_2H_3KO_2$) (≥ 99%), aluminum trinitrate nonahydrate ($Al(NO_3)_3$ x $9H_2O$) (≥ 98%), dimethyl sulfoxide dried, ß–carotene (analytical grade) and *p*–iodonitrotetrazolium violet color (INT) were obtained from Sigma–Aldrich Co., St. Louis, MQ, USA. Sodium carbonate anhydrous (Na_2CO_3) (analytical grade) was purchased from Centrohem d.o.o, Stara Pazova, Serbia. Potassium peroxidisulphate ($K_2O_8S_2$) (≥ 99%), L(+)–ascorbic acid (Vitamin C), Tween 80 and linoleic acid (analytical grade) were obtained from Acros organics, Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK. ABTS (≥ 98%) and quercetin hydrate (≥ 98%) were purchased from TCI Europe NV, Boerenveldsweg, Belgium. Mueller–Hinton agar (MH), malt agar (MA) and Tryptic Soy Broth (TSB) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Streptomycin and ampicilin solutions (100 μg ml^{-1}) were obtained from Hyclone (Logan, Utah, USA). Dimethylsulfoxide (DMSO) (≥ 99.9%) was obtained from Merck KGaA, Germany). Antimicotic Diflucan (containing 50 mg fluconazole) was obtained from Pfizer PGM, Pocesur–Cisse, France.

2.2. Plant material

Plant material was collected at national park Mt. Pelister, surrounding of Bitola city in Republic of Macedonia (GPS: N41°02′21", E21°12′50") in July, 2013. It was determined as *Heracleum orphanidis* Boiss. by one of the authors (Prof. V.S. Matevski). A voucher specimen

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of *H. orphanidis* (BU16777) is deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

2.3. Preparation of plant Es

Pulverized, air–dried plant material (10 g) was treated with 200 ml of different solvents to obtain methanol, ethanol and aqueous Es (ME, EE and AE). The ultrasonic extraction procedure was carried out for 24 h in darkness (the first and the last hour in an ultrasonic bath). Whatman filter paper No.1 was used for filtration after extraction. Methanol and ethanol Es were subjected to solvent evaporation under reduced pressure at maximum temperature of 40ºC. Frozen aqueous Es were lyophilized. Thereafter, the Es packed in glass bottles were measured (Table 1) and kept under refrigeration at 4ºC until further utilization.

2.4. EO isolation

Yellowish EO of *H. orphanidis* was obtained from 200 g of dry plant material, by 3 h of hydrodistilation using Clevenger type apparatus. The yield of the oil was 0.08% for herbal part (w w^{-1}–dry bases). The EO was preserved in a sealed vials at 4ºC prior to the further analysis.

2.5. Extraction method and LC analysis of H. orphanidis MEs of aerial parts and roots with DAD and MS detection

The analysis of the MEs of aerial parts and roots was performed using HPLC–DAD/ESI–ToF–MS system consisting of an HPLC instrument Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany), equipped with a degasser, a binary pump, an auto–sampler, a termostated column compartment and a diode array detector (DAD) and coupled with a 6210 Time–of–Flight LC/MS system (Agilent Technologies, Santa Clara, California, USA) via an electro spray ionization (ESI) interface.

Immediately before analysis, the dry residues of MEs were redissolved in 1 ml acetonitrile–methanol (95:5) and samples (c = 10.0 mg ml^{-1}) were filtered through Captiva Premium Syringe Filter Agilent Technologies (0.45 μm × 25 mm) and in a volume of 5 μl, injected into a Zorbax Eclipse Plus C18 (150 mm × 4.6 mm i.d: 1.8 μm) column, maintained at 40°C. The mobile phase was a mixture of solvent A (0.20% formic acid in water) and solvent B (acetonitrile) according to a combination of isocratic and gradient modes of elution: 0–1.5 min, 95% A, 1.5–26 min, 95–5% A, 26–35 min, 5% A, 36–41 min, 95% A, at a flow rate of 1.40 ml min^{-1}.

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Detection was accomplished using DA detector and storing the signals in the wavelength range from 190–650 nm. The HPLC effluent was directed into the atmospheric pressure ESI ion source of the mass spectrometer. The eluted compounds were mixed with nitrogen in the heated nebulizer interface and the polarity was tuned to positive/negative. An adequate calibration of the ESI parameters (capillary voltage, gas temperature, nebuliser pressure and fragment or voltage) was required to optimise the response and to obtain a high sensitivity of the molecular ion. The MS conditions were as follows: capillary voltage, 4000 V: gas temperature, 350°C: drying gas, 12 ml min^{-1}: nebuliser pressure, 45 psig: fragment or voltage, 140 V: mass range, 100–2000 *m*/*z*. A personal computer system running Mass Hunter Workstation software was used for data acquisition and processing. The Molecular Feature Extractor of Mass Hunter Workstation was used to predict chemical formulas.

2.6. Gas chromatography–flame ionization detector (GC–FID) and gas chromatography–mass spectrometry (GC–MS)

Qualitative and quantitative analysis of the EO was performed using GC–FID and GC–MS methods. The GC–FID analysis of the oil was carried out on a GC HP–5890 II apparatus, equipped with split–split less injector, attached to HP–5 column (25 m × 0.32 mm, 0.52 µm film thickness) and fitted to FID. Carrier gas flow rate (H_2) was 1 ml per min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40–240°C (at rate of 4 ° per min). The same analytical conditions were employed for GC–MS analysis, where HP G 1800C Series II GCD system equipped with HP–5MS column (30 m × 0.25 mm, 0.25 µm film thickness) was used. Transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in *m/z* range 40–400. An identification of the individual EO components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with those held in Wiley 275 library of mass spectra. Confirmation of pick identification was performed using AMDIS software and literature[42]. For the purpose of quantitative analysis area percents obtained by FID was used as a base.

2.7. Antioxidant activity

2.7.1. Determination of DPPH free radical scavenging activity

The free radical scavenging capacity of EO and Es was determinated using DPPH assay described by Blois[43]. This spectrophotometric procedure (JENWAY 6306 UV/Vis) was

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performed to evaluate the quantity of tested solutions needed to reduce 50% of the initial DPPH radical concentration. EO was diluted in methanol (0.25–2.00 µl ml^{-1}), and Es in appropriate solvents (0.25–3.50 mg ml^{-1}). 0.2 ml of each dilution was mixed with 1.8 ml of DPPH methanol solution (0.04 mg ml^{-1}). The absorbance was recorded at a wavelength 517 nm after 30 minutes of dark incubation at room temperature against blank consisting of methanol. BHA and ascorbic acid were used as reference standards. The corresponding percentage of inhibitions of each sample was calculated from obtained absorbance values by using following equation:

Percentage (%) of inhibition = (Ac – As) / Ac x 100.

Tested concentrations of EO and Es which decrease absorption of DPPH solution for 50% (IC_{50}) were obtained from the curve dependence of absorption of DPPH solution on 517 nm from concentration for each tested solution and used standards.

2.7.2. Determination of ABTS radical scavenging activity

The procedure of Miller & Rice–Evans[44] with slightly modifications was followed for determination of *in vitro* ABTS radical scavenging potency of Es. Before usage, 5 ml of the mixture of 2.46 mM potassium persulfate and 19.2 mg of ABTS was allowed to react in the dark for 12–16 h at room temperature to obtain $ABTS^{+}$ solution. 100–110 ml of distilled water was added to 1 ml of $ABTS^{+}$ solution to adjust an absorbance of 0.7 ± 0.02 units at 734 nm. The mixtures of 2 ml of diluted $ABTS^{\cdot+}$ solution and 50 µl of each tested extract solution were incubated for 30 minutes at 30ºC and the absorbance was determined by spectrophotometer at 734 nm, using water as a blank. For every experiment fresh $ABTS^{+}$ solution was prepared.

The results were expressed from Vitamin C calibration curve (0–2 mg l^{-1}) in mg of Vit. C equivalents (E) g^{-1} of dry extract (DE). Tests were carried out in triplicate and all measurements were expressed as average of three analyses ± standard deviation.

2.7.3. The β–carotene bleaching (BCB) test

Antioxidant activity of *H. orphanidis* Es was determined following the *β*–carotene–linoleic acid method described by Miller[45] with some modifications. Methanol solutions of each extract at final concentrations of 2.5 to 15 mg ml^{-1} were incorporated into *β*–carotene–linoleic acid emulsion. The emulsion was prepared by pipetting 2 ml of *β*–carotene solution (2 mg of *β*–carotene was dissolved in 10 ml of chloroform) into covered round bottomed flask containing linoleic acid (40 mg) and Tween 80 (400 mg). The chloroform was evaporated under vacuum at 40°C using a rotary evaporator and after adding 100 ml of oxygenated water, the content was

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vigorously shaken to form an emulsion. Aliquots (2400 µl) of *β*–carotene–linoleic acid emulsion were distributed in test tubes with 100 µl of solutions of tested Es. Zero adjustment was done using blank, consisting of an emulsion without *β*–carotene. The absorbance readings were performed immediately (t = 0 min) at 470 nm using JENWAY 6306 UV/Vis and after incubation for 120 min in a water bath at 50°C. Control samples contained 100 µl of methanol instead of Es mixed with an emulsion. Synthetic references BHT and BHA were also analyzed for comparison. The antioxidant activity of Es was evaluated in term of inhibition of *β*–carotene bleaching caused by radicals formed by linoleic acid oxidation in an emulsion and prevention of its photo–oxidation using the following formula:

Percentage (%) of inhibition = $[(Ac_0 - Ac_{120}) - (As_0 - As_{120}) / Ac_0 - Ac_{120}] \times 100$:

Where Ac_0 and As_0 are the initial absorbance values of control and samples measured at zero time: Ac_{120} and As_{120} are the absorbance values of control and samples after incubation of 120 min. The results are expressed as IC_{50} values (mg ml^{-1}), the concentration required to cause a 50% *β*–carotene bleaching inhibition. Test was carried out in triplicate.

2.7.4. Total phenolic content (TPC)

Spectrophotometric method described by Singleton *et al.*[46] with some modifications was applied for recording total TPCs of all tested Es, using Folin–Ciocalteu reagent and gallic acid (GA) as a standard. After preparing 10% Folin–Ciocalteau reagent, the mixtures of 1000 µl of this solution and 200 µl of Es solutions (1 mg ml^{-1}) were left to react for 6 minutes. After short incubation, 800 µl of 7.5% sodium carbonate solution was added and thus prepared solution was allowed to stand for 2 h at room temperature under condition of darkness. The absorbance was measured at 736 nm versus blank sample. Total phenols were calculated from GA calibration curve (10–100 mg l^{-1}). Data were expressed as milligrams of GAE g^{-1} of DE. The values were presented as means of triplicate analysis.

2.7.5. Total flavonoid content (TFC)

Measurements of TFCs of investigated Es were based on the method described by Park *et al.*[47] with slight modification. An aliquot of each extract solution (1 ml) was mixed with 80% C_2H_5OH, 10% $Al(NO_3)_3$ x $9H_2O$ and 1M $C_2H_3KO_2$. Absorption readings at 415 nm using spectrophotometer were taken after 40 minutes against blank sample consisting of a 0.5 ml 96% C_2H_5OH instead of tested sample. The TFCs were determined from quercetin hydrate (QE)

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standard curve (10–100 mg l^{-1}). Results were expressed as mg of QEE g^{-1} of DE. Measurements were done in triplicates.

2.8. Antimicrobial activity

2.8.1. Preparation of stock solutions of plant Es

H. orphanidis crude Es were dissolved in 5% DMSO to obtain stock solutions (20 mg ml^{-1}) for further testing of antimicrobial activity. Different dilutions of stock Es solutions were tested against different pathogenic microorganisms.

2.8.2. Microbial cultures treated isolates

The antimicrobial activity of all investigated samples was tested using pure control strains obtained from Mycological laboratory, Department of Plant Physiology, Institute for Biologycal Research "Siniša Stanković", Belgrade, Serbia. The microorganisms included following bacterial strains: *Bacillus cereus* (food isolate), *Listeria monocytogenes* (NCTC 7973), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538), *Enterobacter cloacae* (human isolate), *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella typhimurium* (ATCC 13311). The following micromicetes were used: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus niger* (ATCC6275) *Aspergillus ochraceus (*ATCC 12066), *Aspergillus versicolor* (ATCC 11730)*, Penicillium funiculosum* (ATCC 10509), *Penicillium ochrochloron (*ATCC 9112) and *Trichoderma viride* (IAM 5061) and yeast *Candida albicans* (ATCC 10231), Dilutions of bacterial inocula were cultured on solid MH medium, while fungi were maintained on solid MA medium. The cultures were subcultured once a month and stored at + 4°C for further usage[48].

2.8.3. Micro–well dilution assay

For determination of antimicrobial activity of *H. orphanidis* EO and Es, modified microdilution technique described by Hanel and Raether[49] and CLSI, 2009[50] was applied. Assay was performed by sterile 96–well microtiter plates, by adding pure EO or dilutions of tested Es (in 5% DMSO) into corresponding medium – TSB and MA, for bacteria and fungi, respectively. To achieve the concentration of 1.0 x 10^8 colony forming units CFU ml^{-1} for bacterial strains, 100 µl of overnight cultures were mixed with 900 µl of medium in eppendorf. Fungal inocula were prepared by washing spores with sterile 0.85% saline solution (which contains 0.1% Tween 80 (v v^{-1})). The microbial cell suspensions were adjusted with sterile saline to a concentration of approximately 1.0 x 10^6 CFU ml^{-1} for bacteria and 1.0 x 10^5 CFU ml^{-1} for fungi in a final volume

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of 100 µl per well. The microplates were incubated for 24 h at 37°C for bacteria and for 72 h at 28°C for fungi. The lowest concentrations of tested samples completely inhibiting the growth of used pathogens were defined as minimum inhibitory concentrations (MICs). The minimum bactericidal/fungicidal concentrations (MBCs, MFCs) were determined as the lowest concentrations with no visible growth after serial subcultivation, indicating 99.5% killing of the original inoculums[49]. In addition, bacterial growth was determined by a colorimetric microbial viability assay, based on reduction of 0.2% INT aqueous solution and compared with positive control for each bacterial strain[50,51]. Two replicates were done for each sample. The solution of synthetic standard streptomycin with concentration of 1 mg ml^{-1} 5% DMSO was used as positive control for bacteria, while the fluconazole solution at concentration of 2 mg ml^{-1} 5% DMSO, was included for fungi. Sterilized distilled water containing 0.02% Tween 80 and 5% DMSO was used as negative control.

2.9. Anti–QS activity of EO and Es

2.9.1. Bacterial strains, growth media and culture conditions

In this work, *Pseudomonas aeruginosa* PAO1 from the collection from Mycoteca of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, was used. Bacteria were routinely grown in Luria–Bertani (LB) medium (1% w v^{-1} NaCl, 1% w/v Tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37°C.

2.9.2. Biofilm formation

The effect of different concentrations of the EO and ME, EE and AE of *H. orphanidis* (ranging from 0.5, 0.25 and 0.125 of MICs, respectively) on biofilm forming ability was tested on polystyrene flat–bottomed microtitre 96 – well plates as described by Spoering and Lewis[52]: Drenkard and Ausubel[53], with some modifications.

In brief, 100 µl of overnight culture of *P. aeruginosa* (inoculum size was 1 x 10^8 CFU ml^{-1}) was added to each well in the presence of 100 µl subinhibitory concentrations (subMIC) of *H. orphanidis* samples (0.5, 0.25 and 0.125 MIC) or 100 ml medium (control). After incubation for 24 h at 37ºC, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. After drying, 200 µl of 95% ethanol (v v^{-1}) was added to solubilise the dye that had stained the biofilm cells. The excess stain was washed off with dH_2O. After 10 min, the content of the wells was homogenized and the

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absorbance at λ = 625 nm was read on a Sunrise™ – TecanELISA reader. The experiment was done in triplicate and repeated two times and values were presented as a mean values ± SE.

2.9.3. Twitching and flagella motility

After growth in the presence or absence of subMICs of *H. orphanidis* samples and reference controls, the cells of *P. aeruginosa* PAO1 were washed twice with sterile PBS and re–suspended in PBS at 1 x 10^8 CFU ml^{-1} (OD of 0.1 at 660 nm). In brief, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37°C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope[54,55]. SubMIC (0.5 MICs) of EO and Es were mixed into 10 ml of molten MH medium and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of PAO1 once the overlaid agar had solidified and incubated at 37°C for 3 days. The extent of swimming was determined by measuring the area of the colony[56]. Experiment was done in triplicate and repeated two times. The colony diameters were measured three times in different direction and values were presented as a mean values ± SE.

2.9.4. Inhibition of synthesis of P. aeruginosa PAO1 pyocyanin

The flask assay was used to quantify the inhibitory activity of the *H. orphanidis* against *P. aeruginosa* pyocyanin production. Overnight culture of *P. aeruginosa* PAO1 was diluted to OD_{600} nm 0.2. Then, 0.5 MICs of tested EO (0.065 mg ml^{-1}) and Es dissolved in 5% of DMSO (0.5 – 2.5 mg ml^{-1}) were added to the bacteria (5.00 ml) and incubated at 37°C for 24 h. The treated culture was extracted with chloroform (3 ml), followed by mixing the chloroform layer with 0.2 M HCl (1 ml). Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan) [56]. Experiment was done in triplicate and repeated two times. The values were expressed as ratio (OD_{520} / OD_{600}) x 100.

2.10. Statistical analysis

For tested EO and each extract, three samples were used and all assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one–way analysis of variance (ANOVA) followed by Tukey's HSD Test with a = 0.05. This analysis was carried out using SPSS v. 18.0 program.

3. Results

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3.1. EO chemical composition

According to GC–FID and GC–MS, fifty–five constituents were determined in EO of *H. orphanidis*, representing 97.24% of the total EO amount. The components are listed in Table 2, and a significant presence of fatty acids derivates (80.78%) is evident. The EO consisted mainly of fatty alcohols (40.98%), fatty acid esters (39.80%) and carbonylic compounds (16.46%) Sesquiterpene hydrocarbons (4.63%) were the most abundant group of carbonylic compounds. Also, it was noted that aldehydes prevailed over ketones, with *n*–octanal (1.20%) predominated, while oplopanone (2.12%) was the most abundant ketone. The most dominant constituent in the EO was fatty alcohol *n*–octanol (39.57%), followed by esters of fatty acids, octyl hexanoate (17.62%) and *n*–octyl acetate (14.12%).

3.2. Tentative analyzes of MEs

Tentatively assigned components of the MEs of the aerial parts and the roots of *H. orphanidis* are given in Tables 3 and 4, respectively. The identification of components was based on LC–MS analysis and they were assigned in concordance with the molecular formula and supported by LC–DAD data. The MEs of aerial parts and the roots of *H. orphanidis* are characterised by furanocoumarins. Ten linear and five angular furanocoumarins, beside the coumarin dimer moellendorffilin and three furanocumarin glucosides (apterin, yunngnoside B, smyrindioloside) were identified in the root extract (Table 4). Eight furanocoumarins and four simple coumarins (6,7,8–trimethoxycoumarin, 7–isopentenyloxycoumarin, osthenol and ostruthin) as well as 5–sinapoylquinic acid were recognized in the aerial parts extract. *E,E*–Farnesylacetone, linolenic, pinellic, vernolic and 9*Z*,12*Z*–linoleic acids were identified in both MEs. The well known polyacetylenic ketone falcarinone was found in the aerial parts of the plant. Two components were identified for the first time in Apiaceae family: 5–sinapoylquinic acid, detected in aerial parts and vernolic acid detected in both extracts. The commonest furanocoumarins of the genus *Heracleum* such as isobergapten, bergapten, sphondin, pimpinellin, isopimpinellin and xanthotoxin were identified in both MEs of this species (Tables 3 and 4).

3.3. Antioxidant activity, TPCs and TFCs

Table 5 presents the results of antioxidant activities of *H. orphanidis* samples carried out by three different assays. According to DPPH test, the best radical scavenging potential showed ME of aerial parts (IC_{50} = 0.55 mg ml^{-1}). The antioxidant activities of tested samples were

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recorded in following order: ME > EE > AE > EO for aerial parts Es and EE > ME > AE for roots Es. The results of ABTS test suggested that AE of aerial parts (1.13 mg Vit. C g^{-1} of DE) had the highest activity, as twice as effective then ME of aerial parts (0.642 Vit. C g^{-1} of DE). Due to *β*–carotene–linoleic acid test, the results were consistent with the data obtained in DPPH test. The inhibition potential of *H. orphanidis* ME of aerial parts (4.28 mg ml^{-1}) was found to be the highest, contrary to AE of roots (17.46 mg ml^{-1}). AE of aerial parts was registered as the sample with the highest phenolics content, while measured values of total flavonoid contents ranged from 1.77 to 24.23 mg QE g^{-1} for dry EEs of aerial part and roots, respectively.

In general, higher phenolic and flavonoid contents recorded in Es obtained from aerial parts, resulted in stronger antioxidant potential detected by all applied assays (Table 5). According to *β*–carotene bleaching test, MEs expressed stronger antioxidant potential than EEs, while in DPPH and ABTS assays their activities were similar. It was observed that AE of roots revealed the weakest antioxidant activity and the lowest concentration of total phenols.

3.4. Antimicrobial properties

Antimicrobial activities of *H. orphanidis* samples are listed in Tables 6 and 7. Regarding to antibacterial activity of *H. orphanidis*, inhibition of growth of Gram–positive strains was more prominent. As presented in Table 6, EO has proven to be the most effective against used bacteria (MBCs = 0.26 – 1.05 mg ml^{-1}), showing the similar activity to streptomycin. The most sensitive strains were *B. cereus* and *S. typhimurium*, while *M. flavus*, *L. monocytigenes* and *E. coli* revealed to be the most resistant bacteria (Table 6). According to the results shown in Table 7, the strongest antifungal potential was reached with ME of roots (MFCs = 3 – 14 mg ml^{-1}), which was still lower than control fluconazole activity. The most sensitive fungus was *A. versicolor*, while *A. niger* and *C. albicans* were the most resistant fungi. According to values of effective concentrations, it can be concluded that Es exhibited higher antibacterial than antifungal potency.

3.5. Anti–QS activity of EO and Es

3.5.1. Biofilm formation

The results of *H. orphanidis* EO and Es effects on *P. aeruginosa* PAO1 biofilm formation are presented in Table 8. The samples were tested at 0.5, 0.25 and 0.125 of MIC values (MICs are given in Table 6). It was demonstrated that subMICs of used samples were sufficient for effective reduction and inhibition of the biofilm formation. All tested samples showed certain anti–biofilm activity, but the best reduction was noticed in the presence of 0.125MICs of the

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samples. *H. orphanidis* allowed the biofilm formation in the range of 52.66 % – 81.64 %, where EE of aerial parts had the strongest inhibition effect on biofilm formation, reducing 32.13% to 47.34% of biofilm formation. The lowest activity showed EO, which reduced only 18.33 – 19.08% of colony formation. In comparison, in the presence of commercial antibiotics streptomycin and ampicillin, biofilm formation occurred in wider range (49.40 % – 92.16 %), with slightly stronger biofilm inhibition recorded for streptomycin.

3.5.2. Twitching and flagella mobility

In this study, *H. orphanidis* samples reduced the twitching and flagella mobility of *P. aeruginosa*. As it can be seen from Table 9, the colors of the colonies ranged from white, through light green to green. After two days of incubation at room temperature, expansion of control colony occurred very rapidly due to twitching mobility. Due to Table 9 and Fig. 1, control colony produced swimming zones to 100% with diameter of 12.00 mm and displayed rough appearance, regular surface, irregular colony edges and a hazy zone around the colony in the absence of tested EO and Es. The flagella were with regular size and the cells were in very thin layer.

Bacterial colonies grown in the presence of *H. orphanidis* samples were capable of producing twitching zone with round, smooth, regular colony edges. Colonies in the presence of aerial parts Es were smaller in diameter than in the presence of roots Es. The largest in diameter were colonies treated with ME and EE of aerial parts (20.33 mm and 18.00 mm, respectively), contrary to colonies treated with EO and streptomycin (10.33 mm and 11.00 mm, respectively).

Considering shape, number and size of flagella in bacterium, the most reduced flagella in all tree parameters were in colony grown with roots AE. Contrary, the largest, almost regular flagella were detected in the presence of ME of aerial parts. Streptomycin reduced the flagella in a large degree, while ampicillin minimally affected the formation of flagella (Fig. 1, Table 9).

3.5.3. Inhibition of synthesis of P. aeruginosa PAO1 pyocyanin

A flask assay was used to quantify *H. orphanidis* QS inhibitory activity against pyocyanin production. Addition of subMICs of EO and Es effected production of pyocyanin in certain amount, the presence of which is indicated by the green coloration of the samples. Referring to the results from Fig. 2 it could be seen that pyocyanin was less produced in the presence of EO and Es (from 41.07% to 102.97%) than by control strain (141.55%). It was observed that addition of some *H. orphanidis* samples reduced the level of the pigmentation

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more than in the presence of used antibiotics. The strongest inhibition of pigment's production was detected by EO and EE of aerial parts. *H. orphanidis* EO (41.03%), EEs of aerial parts and roots (47.20 and 83.40%, respectively) and AE of roots (64.52%) more efficiently prevented pigment production than both applied antibiotics (84.27% and 97.56% for streptomycin and ampicillin, respectively). The maximum of pyocyanin synthesis was detected with addition of ME of aerial parts and roots, followed by AE of aerial parts. Inhibition of pyocyanin production of *P. aeruginosa* by *H. orphanidis* samples has further strengthened its anti–QS behavior.

4. Discussion

EO composition of *H. orphanidis* reported in this study revealed *n*–octanol, *n*–octyl acetate and octyl hexanoate as the most abundant compounds. Octyl acetate, present in notable amount in *H. orphanidis* oil, previously was identified as the major component of *H. crenatifolium*, *H. sphondylium* and *H. platytaenium* oil samples, while octyl acetate and *n*–octanol were dominant in *H. crenatifolium* EO subsequently studied[18,57]. Similarly to the results obtained for this EO, the main compounds in *H. sphondylium* EO were *n*–octanol, octyl–butirate and octyl–acetate[14,58]. In some recorded data, it has been shown that *H. persicum* EO was characterized by hexyl butyrate, octyl acetate and also by viridiflorol and *trans*–anethole which were completely absent in *H. orphanidis* sample[6,29,59,60]. Additionally, components like myristicin, (*E*)–anethole, hexyl butanoate and elemicin were not detected in this study, although some authors cited them as major components in related *Heracleum* species[6,61,62].

Specified structures of components tentatively assigned in *H. orphanidis* MEs are mostly in line with literature on *Heracleum* species or family Apiaceae[63–98]. The most abundant components in the aerial parts and the roots of *H. orphanidis* are furanocoumarins, which are characteristic for the genus and could be find in all plant parts (aerial parts, roots, seeds) [8,20,25,67,68]. The polyacetylenic ketone falcarinone was found in the aerial parts. This compound is characteristic for Apiaceae vegetables[69,70], but there is no data about its presence in the genus *Heracleum.* 5–sinapoylquinic acid[71] and vernolic acid[72] have not been identified in the family Apiaceae so far. On the contrary, all determined coumarins were found in the *Heracleum* species. Isobergapten, bergapten, sphondin, pimpinellin, isopimpinellin and xanthotoxin[73–75] occur in 30 – 40 species at least, representing the commonest furanocoumarin of the genus.

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Previously, some studies on *in vitro* antioxidant and antimicrobial activity of related species were published. According to the results presented in Table 5, it can be concluded that *H. orphanidis* EO showed moderate antioxidant activity, which is in accordance to the results of Firuzi *et al*.[6] where *H. pastinacifolium, H. transcaucasicum*, *H. rechingeri* and *H. persicum* EOs demonstrated moderate antioxidant effects. Also, the modest antioxidant potential of *H. transcaucasicum* EO was shown by Torbati *et al*.[61] In this study, *H. orphanidis* EO showed significantly lower antioxidant activity compared to ascorbic acid and BHA, contrary to *H. sprengelianum* EO which expressed higher effect than these controls[13]. Furthermore, it was noted that all tested *H. orphanidis* samples exhibited lower radical scavenging activity compared to used references. Analyses presented in this work showed that ME of roots possessed low antioxidant activity (Table 5), opposite to the results obtained by Dash *et al*.[15] for ME of *H. nepalense* roots which exhibited significant potency. Approximate intensity of antiradical activity was observed for MEs of aerial parts of *H. orphanidis* and *H. persicum*[31]. In addition, all *H. orphanidis* Es exhibited moderate to low lipid peroxidation inhibitory activity compared to used references: the same results in *β*–carotene bleaching method were demonstrated for *H. platytaenium* petroleum ether and methanol Es[20].

H. orphanidis EO, MEs and EEs exhibited significant anti–staphylococal activity in the same range as *H. nepalense* Es of roots in the study of Dash *et al*.[15] Considering antibacterial activity, *H. orphanidis* EO was more effective compared to *H. sphondylium* EO[13]. While *H. orpanidis* EO stopped the growth of *P. aeruginosa* in the concentration less than streptomycin (Table 6), it was the most sensitive strain in the work of Firuzi *et al*.[6] The findings achieved for *H. anisactis* and *H. transcaucasicum* EOs and *H. sphondylium* Es showed that they were ineffective against used bacteria[33,61]. *H. orphanidis* AEs expressed low activity towards some of tested bacteria and similar results were recorded for AEs of *H. candicans*, *H. persicum* and *H. mantegazzianum*[99,100].

Although, some authors have reported inhibitory anti–QS effects of plants and compounds from natural sources[35,38–40,101], *H. orphanidis* was not examined for its anti–QS activity. Results obtained for anti–QS activity of *H. orphanidis* as natural agent against pathogenic *P. aeruginosa* are of significant value since virulence factors, regulated by gene expression are the mode of bacteria's protection from the immune system of the host[36]. An essential role in the initial stage of biofilm formation is played by twitching and flagella mobility

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of *P. aeruginosa*, performed by specific flagellum and type IV pili. Toxic pigment pyocynin produced by this bacterium acts as a virulence factor and reduction of its production is crucial for increasing the effectiveness of host defense[55]. To the present, there is no sufficient information that can point out a genuine mechanism of QS inhibition. Earlier, a few potential modes of action have been proposed based on interference with the QS system, such as inhibition of biosynthesis of auto–inducer molecules, inactivation or degradation of the auto–inducer, interference with the signal receptor and inhibition of the genetic regulation system[102].

Considerable efforts were made for discovering new, natural anti–QS agents. Among MEs of 97 plants from Korea that were screened for anti–QS activity, *Angelica dahurica* (Apiaceae) was in group of sixteen species that possessed the strongest anti–QS activity against *P. aeruginosa* PAO1 and *C. violaceum* CV12472[103]. Chong *et al*.[104] also found that *A. dahurica* roots Es exhibited anti–QS properties, by inhibiting the selected virulence determinants of *P. aeruginosa* PAO1. EOs of *Ferula asafoetida* and *Dorema aucheri* (Apiaceae), exhibited anti–QS activity against *P. aeruginosa*, but pyocyanin and biofilm production were not affected by *D. aucheri* EO[35]. In our study, *H. orphanidis* EO had the best reduction potential in suppression of pyocianin synthesis. Furocoumarins recorded in this work as the main group of compounds in *H. orphanidis* MEs, were shown to hinder the formation of biofilm in *P. aeruginosa E. coli* and *S. typhimurium*[105,106]. Opposite results in colony diameter and anti–biofilm activity of *H. orphanidis* EO and EE of aerial parts were observes. It can be seen from Tables 6 and 8 that antibacterial and anti–biofilm activities of EO were in contrast as well. The results obtained for these samples could be associated with possible different mechanisms responsible for their activity. Different ingredients could be associated with specific effects on QS system due to complexity of EO and Es compositions. In addition, various plant Es differently affect the biofilm formation, so the method of preparation of Es should be also considered[38,107]. Obtained results for *H. orphanidis* suggested that AEs expressed less anti–biofilm activity compared to MEs and EEs, which was in conformity with results obtained for antimicrobial activity. Es obtained from aerial parts were more effective than Es obtained from roots in suppression of biofilm formation (Table 8). All this data additionally validate the use of higher plants as native source rich in anti–QS compounds as novel virulence inhibitors.

5. Conclusions

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Given results revealed that EO contained fifty–five compounds in various concentrations with *n*–octanol as the main component and this identification provided the possibility for consideration towards future focus on targeting the active constituents and their mutual interactions. The tentative examination of *H. orphanidis* MEs was done to obtain a preliminary profile of variety of their compounds. This underlines the potential of *H. orphanidis* as an exploitable source for isolation of its pure compounds, which would valorize this species. Tested samples possessed antioxidant activity, especially ME and AE of aerial parts. Since EO has proven to be the most effective against used bacteria, this indicates potential use of *H. orphanidis* in prevention or/and in control of microbial growth. Thus, these species may become pharmaceutically relevant, particularly for antibiotic–resistant strain *P. aeruginosa*. The results encourage the application of plant for further evaluations of other possible bioactivities and detection of active pure compounds as constituents of the EO and Es in order to confirm these findings. Taken together, data presented in this study highlighted the potential of *H. orphanidis* as source of natural material for therapeutic use.

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Conflicts of interest

There are no conflicts of interest to declare.

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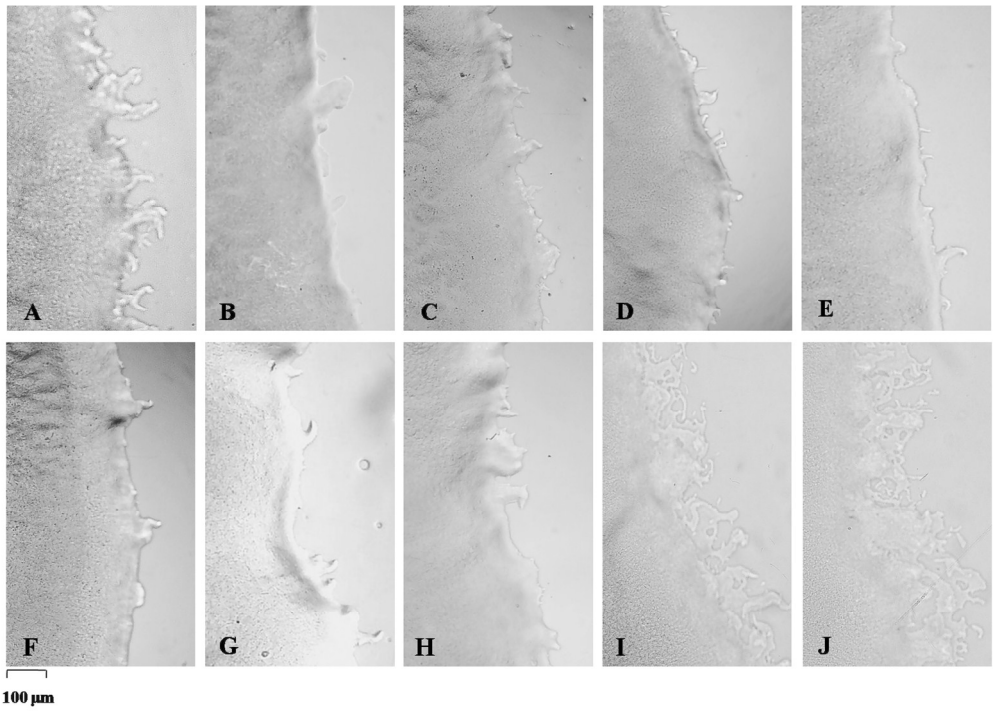

Fig. 1 Light microscopy of colony edges of P. aeruginosa in twitching motility, grown in the presence or absence of H. orphanidis EO, Es and commercial antibiotics. The bacterial colonies grown with the presence of 0.5 MIC of Es (A–F) and EO (G); P. aeruginosa colony in presence of streptomycin (0.5 MIC) had reduced protrusion (H); P. aeruginosa colony in presence of ampicillin with regularly formed protrusions (I); P. aeruginosa produced a flat, widely spread, irregularly shaped colony in the absence of EO and Es (J); Magnification: (A–D) × 100.

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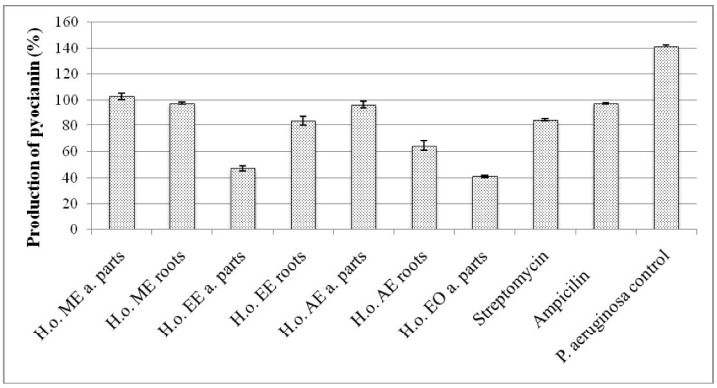

Fig. 2 Reduction of pyocyanin production of P. aeruginosa PAO1 by H. orphanidis EO, Es and streptomycin and ampicillin tested at subMICs (mg ml–1).

279x361mm (300 x 300 DPI)

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Table 1 Yields of *H. orphanidis* crude Es obtained by ultrasonic extraction (g)

	***H. orphanidis* Es**					
	ME		EE		AE	
	Aerial parts	Roots	Aerial parts	Roots	Aerial parts	Roots
Mass (g)	0.686	2.149	0.510	1.341	0.538	1.417

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29 **Table 2** Chemical composition of EO of *H. orphanidis* aerial parts

Compounds	**KIE***	**KIL***	**%**
n-Heptanal	911.9	901	0.09
Sabinene	966.5	969	0.21
2-Pentyl furan	984.9	984	0.10
n-Octanal	998	998	1.20
***n*-Octanol**	1090.8	1063	**39.57**
n-Nonanal	1099	1100	0.77
cis-p-Menth-2-en-1-ol	1121.4	1118	0.12
trans-p-Menth-2-en-1-ol	1138	1136	0.11
trans-Verbenol	1142.9	1140	0.09
p-Mentha-1,5-dien-8-ol	1165.1	1166	0.08
Terpinen-4-ol	1171.7	1174	0.45
p-Cymen-*8-ol*	1187.9	1179	0.24
***n*-Octyl acetate**	1210.3	1211	**14.12**
n-Decanol	1266.1	1266	1.41
Isobornyl acetate	1271.6	1283	0.20
Undecanal	1294.2	1305	0.29
α-Copaene	1357.9	1374	0.16
Daucene	1361.7	1380	0.18
b-Bourbonene	1366.5	1387	0.30
β-Elemene	1378.1	1389	2.03
Dodecanal	1395.8	1408	0.24
Decyl acetate	1397.5	1407	0.76
trans-Caryophyllene	1402.3	1417	0.47
n-Octyl 2-methyl butyrate	1425.2	1421	1.39
α-Humulene	1434.3	1452	0.17
trans-β-Farnesene	1440.5	1454	0.16
Germacrene D	1462.5	1484	0.49
β-Selinene	1467.3	1489	0.34
(*E*)-*β*-Ionone	1469.5	1487	0.42
α-Selinene	1477.4	1498	0.33
n-Octyl 3-methyl-2-butenoate	1504.5	n.i.*	0.47
trans-Nerolidol	1560.1	1561	0.98
Octyl hexanoate	1581.7	1570	**17.62**
Carotol	1589.7	1594	0.30
Tetradecanal	1596.9	1611	0.18
Hinesol	1637.8	1640	0.18
Selina-3,11-dien-6-*α*-ol	1641.0	1642	0.19
Cedr-8(15)-en-10-ol	1650.3	1650	0.13
Cedr-8(15)-en-9-*α*-ol	1655.3	1650	0.16
8-Cedren-13-ol	1690.1	1688	0.13
epi-Nootkatol	1703.7	1699	0.11
Oplopanone	1745.4	1739	2.12
Octyl octanoate	1763.8	1767	1.91

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Octyl benzoate	1799.3	1792	0.13
Neophytadiene, Isomer II	1822.8	1830	1.36
Hexahydrofarnesyl acetone	1828.6	1845	0.22
Neophytadiene, Isomer III	1846.1	1854	0.03
Octyl nonanoate	1856	1876	0.13
Nonadecane	1884.9	1900	0.06
Methyl hexadecanoate	1917.1	1921	0.12
Octyl decanoate	1963.4	1978	2.55
Octadecanal	2017.1	2017	0.48
trans-Phytol	2091.5	2114	0.79
Octyl dodecanoate	2150.1	2158	0.14
Octyl tetradecanoate	2348.8	2374	0.26
Total			**97.24**
Number of constituents			55
Monoterpene hydrocarbons			0.21%
Oxygenated monoterpenes			1.09%
Sesquiterpene hydrocarbons			4.63%
Oxygenated sesquiterpenes			2.18%
Oxygenated diterpenes			0.79%
Aldehydes			3.25%
Esters of fatty acids			39.8%
Fatty alcohols			40.98%
Ketones			2.54%
Others			1.77%

*KIE: Kovàts (retention) Index experimentally determined (AMDIS).

*KIL: Kovàts (retention) Index - literature data (Adams, 2007).

*n.i.: not identified

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Table 3 Preliminary LC-MS analysis of the composition of ME of *H. orphanidis* aerial parts

R_t (min)	TOFMS (*m/z*) [ESI^+]	Formula	Compound	Reference
7.968	416.1543 $[M + NH_4]^+$	$C_{18}H_{22}O_{10}$	5-Sinapoylquinic acid	Schinkovitz et al. (2003)
	237.0756 $[M + H]^+$	$C_{12}H_{12}O_5$	6,7,8-Trimethoxycoumarin	Williams (1970)
12.425	217.0505 $[M + H]^+$	$C_{12}H_8O_4$	Isobergapten, Bergapten, Allobergapten, Sphondin	Niu et al. (2002)
13.395	217.0520 $[M + H]^+$	$C_{12}H_8O_4$	Xanthotoxin	Niu et al. (2002), Gupta et al. (1978)
13.495	247.0598 $[M + H]^+$,	$C_{13}H_{10}O_5$	Pimpinellin, Isopimpinellin	Niu et al. (2002), Zhongwen et al. (1993)
14.095	158.1533 $[M + H]^+$ 315.3008 $[2M + H]^+$	$C_9H_{19}NO$	Not identified	-
17.266	231.1072 $[M + H]^+$, 483.1774 $[2M + Na]^+$	$C_{14}H_{14}O_3$	7-Isopentenyl-Oxycoumarin, Osthenol	Belenovskaya et al. (1977) Komissarenko and Buziashvili (1966)
18.034	301.1062 $[M + H]^+$	$C_{17}H_{16}O_5$	Phellopterin	Marčetić et al. (2013), Roman et al. (2011)
20.063	279.2326 $[M + H]^+$	$C_{18}H_{30}O_2$	Linolenic acid	Maggio et al. (2013), Fischer and Svendsen (1976)
22.771	299.1651 $[M + H]^+$	$C_{19}H_{22}O_3$	Ostruthin	Schinkovitz et al. (2003), Williams (1970)
23.651	280.2674 $[M + NH_4]^+$	$C_{18}H_{30}O$	*E,E*-Farnesylacetone	Inoue et al. (2010)
12.656	329.2344 $[M - H]^-$,	$C_{18}H_{34}O_5$	Pinellic acid	Waksmundzka-Hajnos et al. (2006),
20.050	295.2286 $[M - H]^-$, 331.2057 $[M + Cl]^-$,	$C_{18}H_{32}O_3$	Vernolic acid	Belenovskaya et al. (1977)
25.740	279.2336 $[M - H]^-$,	$C_{18}H_{32}O_2$	9*Z*,12*Z*-Linoleic acid	Belenovskaya et al. (1977)

Mass accuracy within 5 ppm

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Table 4 Preliminary LC-MS analysis of the composition of ME of *H. orphanidis* roots

R_t (min)	TOFMS (*m/z*) [ESI^+/ESI^-]	Formula	Compound	Reference
6.973	263.0915 $[M + H]^+$	$C_{14}H_{14}O_5$	Vaginidiol, Smyrindiol	Shimomura et al. (1982), Merijanian et al. (1980), Gupta et al. (1976) Jimenez et al. (2000)
9.993	287.0912 $[M + H]^+$	$C_{16}H_{14}O_5$	Heraclenin, Isogosferol	Gupta et al. (1978) Bandopadhyay and Seshadri (1970), Kumar et al. (1976), Pathak and Manral (1988), Razdan et al. (1982)
	322.1281 $[M + NH_4]^+$	$C_{16}H_{16}O_6$	Heraclenol	Rastogi et al. (2007), Saraswathy et al. (1990), Kasumova and Serkerov (2011)
12.350	217.0496 $[M + H]^+$	$C_{12}H_8O_4$	Isobergapten, Bergapten, Allobergapten, Sphondin	Niu et al. (2002)
13.389	217.0520 $[M + H]^+$	$C_{12}H_8O_4$	Xanthotoxin	Niu et al. (2002), Gupta et al. (1978)
13.496	247.0790 $[M + H]^+$, 515.0945 $[2M+Na]^+$	$C_{13}H_{10}O_5$	Pimpinellin	Niu et al. (2002), Zhongwen et al. (1993)
14.168	247.0599 $[M + H]^+$	$C_{13}H_{10}O_5$	Isopimpinellin	Niu et al. (2002), Zhongwen et al. (1993)
17.329	271.0972 $[M + H]^+$	$C_{16}H_{14}O_4$	Imperatorin, Isoimperatorin	Gupta et al. (1978), Komissarenko et al. (1978)
17.462	493.1125 $[M + H]^+$, 510.1392 $[M + NH_4]^+$, 1007.2003 $[2M + Na]^+$	$C_{26}H_{20}O_{10}$	Moellendorffilin	Komissarenko et al. (1978), Chacko et al. (2001)
18.033	301.1069 $[M + H]^+$	$C_{17}H_{16}O_5$	Phellopterin	Marčetić et al. (2013), Roman et al. (2011)
19.864	243.1747 $[M + H]^+$ 485.3412 $[2M + H]^+$	$C_{17}H_{22}O$	Falcarinone	Tolibaev and Glushenkova (1996), Makarenko et al. (2007)
20.050	279.2318 $[M + H]^+$	$C_{18}H_{30}O_2$	Linolenic acid	Maggio et al. (2013), Fischer and Svendsen (1976)
23.657	280.2727 $[M + NH_4]^+$	$C_{18}H_{30}O$	*E,E*-Farnesylacetone	Inoue et al. (2010)
7.446	459.1069 $[M + Cl]^-$, 469.1360 $[M + HCO_2]^-$	$C_{20}H_{24}O_{10}$	Apterin, Yunngnoside B, Smyrindioloside	Kobayashi et al. (1990), Tsevegsuren et al. (2004), Lin and Harnly (2008)
12.656	329.2344 $[M - H]^-$,	$C_{18}H_{34}O_5$	Pinellic acid	Waksmundzka-Hajnos et al. (2006)
20.050	295.2286 $[M - H]^-$, 331.2057 $[M + Cl]^-$,	$C_{18}H_{32}O_3$	Vernolic acid	Belenovskaya et al. (1977)
25.740	279.2336 $[M - H]^-$,	$C_{18}H_{32}O_2$	9*Z*,12*Z*-Linoleic acid	Komissarenko and Buziashvili (1966)

Mass accuracy within 5 ppm

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Table 5 Antioxidant activities, TPCs and TFCs of *H. orphanidis* Es and EO (means ± SD)

Assay/ *H. orphanidis* Es/EO/Standards		DPPH (IC_{50} = mg ml^{-1})	ABTS 1 mg/ml (mg Vit. C g^{-1} of DE)	B carotene linoleic acid (IC_{50} = mg ml^{-1})	TPC 1 mg/ml (mg GA g^{-1} of DE)	TFC 1 mg ml (mg QE g^{-1} of DE)
ME	Aerial parts	0.55 ± 0.01^{b}	0.64 ± 0.004^{c}	4.28 ± 0.01^{ab}	57.20 ± 0.002^{b}	18.39 ± 0.007^{b}
	Roots	1.86 ± 0.02^{c}	0.26 ± 0.002^{d}	7.78 ± 0.02^{b}	33.36 ± 0.007^{c}	2.23 ± 0.000^{c}
EE	Aerial parts	0.67 ± 0.01^{b}	0.61 ± 0.002^{c}	6.59 ± 0.01^{b}	57.21 ± 0.002^{b}	24.23 ± 0.004^{a}
	Roots	1.76 ± 0.01^{c}	0.21 ± 0.000^{d}	9.86 ± 0.01^{c}	35.55 ± 0.006^{c}	1.78 ± 0.005^{c}
AE	Aerial parts	0.70 ± 0.09^{b}	1.13 ± 0.000^{b}	8.51 ± 0.03^{bc}	75.75 ± 0.004^{a}	16.15 ± 0.007^{b}
	Roots	2.81 ± 0.03^{d}	0.20 ± 0.001^{d}	17.46 ± 0.02^{d}	28.74 ± 0.002^{c}	5.08 ± 0.005^{c}
EO		2.02 ± 0.02^{c}	n.d.*	n.d.	n.d.	n.d.
Standards		BHA 0.13 ± 0.01^{a} Vit C. 0.03 ± 0.09^{a}	QE 2.75 ± 0.004^{a}	BHA 1.22 ± 0.02^{a} n.d.	n.d.	n.d.

Indicated letters mean significant difference ($p < 0.05$)

*n.d.: not determinate

Table 6 Antibacterial activities of *H. orphanidis* and standard determinated by microdilution method in mg ml^{-1} (means ± SD)

Bacteria/			Gram-positive bacteria				Gram-negative bacteria			
***H. orphanidis* Es/EO/Standard**			***B. cereus***	***M. flavus***	***L. monocytogenes***	***S. aureus***	***P. aeruginosa***	***E. coli***	***En. cloacae***	***S. tiphymurium***
ME	Aerial parts	**MIC**	0.4 ± 0.01^{a}	2 ± 0.06^{b}	1 ± 0.07^{a}	0.4 ± 0.03^{a}	2 ± 0.10^{b}	4 ± 0.01^{c}	2 ± 0.03^{b}	2 ± 0.00^{b}
		MBC	1 ± 0.07^{a}	4 ± 0.03^{b}	2 ± 0.01^{a}	1 ± 0.00^{a}	3 ± 0.07^{b}	7 ± 0.10^{c}	4 ± 0.04^{b}	3 ± 0.00^{b}
	Roots	**MIC**	0.2 ± 0.01^{a}	1 ± 0.01^{b}	1 ± 0.00^{b}	0.5 ± 0.00^{a}	1 ± 0.04^{b}	0.5 ± 0.05^{a}	2 ± 0.03^{c}	1 ± 0.05^{b}
		MBC	0.5 ± 0.03^{a}	2 ± 0.01^{a}	4 ± 0.01^{b}	1 ± 0.01^{a}	4 ± 0.02^{b}	1 ± 0.01^{a}	6 ± 0.00^{c}	2 ± 0.04^{a}
EE	Aerial parts	**MIC**	0.2 ± 0.02^{a}	2 ± 0.07^{b}	1 ± 0.02^{ab}	1 ± 0.05^{ab}	1 ± 0.02^{ab}	4 ± 0.00^{c}	2 ± 0.01^{b}	0.5 ± 0.01^{a}
		MBC	0.3 ± 0.02^{a}	4 ± 0.03^{c}	2 ± 0.02^{b}	2 ± 0.01^{b}	3 ± 0.05^{b}	5 ± 0.03^{c}	3 ± 0.08^{b}	1 ± 0.08^{a}
	Roots	**MIC**	0.4 ± 0.07^{a}	2 ± 0.02^{b}	2 ± 0.09^{b}	0.3 ± 0.03^{a}	2 ± 0.01^{b}	0.4 ± 0.03^{a}	0.5 ± 0.01^{a}	0.5 ± 0.03^{a}
		MBC	0.5 ± 0.01^{a}	5 ± 0.07^{b}	8 ± 01^{c}	0.4 ± 0.02^{a}	8 ± 0.01^{c}	0.5 ± 0.01^{a}	1 ± 0.01^{a}	1 ± 0.00^{a}
AE	Aerial parts	**MIC**	5 ± 0.09^{a}	10 ± 0.07^{b}	5 ± 0.05^{a}	10 ± 0.07^{b}	5 ± 0.00^{a}	10 ± 0.04^{b}	10 ± 0.00^{b}	10 ± 0.01^{b}
		MBC	> 14 ± 0.05^{c}	> 14 ± 0.05^{c}	14 ± 0.1^{b}	> 14 ± 0.05^{c}	11 ± 0.04^{a}	13 ± 0.07^{b}	14 ± 0.05^{b}	> 14 ± 0.08^{c}
	Roots	**MIC**	5 ± 0.03^{a}	10 ± 0.01^{c}	5 ± 0.02^{a}	5 ± 0.00^{a}	5 ± 0.07^{a}	8 ± 0.02^{b}	5 ± 0.02^{a}	5 ± 0.02^{a}
		MBC	9 ± 0.01^{a}	> 14 ± 0.05^{c}	11 ± 0.01^{b}	7 ± 0.02^{a}	11 ± 0.02^{b}	9 ± 0.01^{a}	10 ± 0.01^{b}	10 ± 0.02^{b}
EO		**MIC**	0.13 ± 0.03^{a}	0.26 ± 0.01^{b}	0.26 ± 0.00^{b}	0.13 ± 0.04^{a}	0.13 ± 0.03^{a}	0.26 ± 0.07^{b}	0.26 ± 0.02^{b}	0.13 ± 0.07^{a}
		MBC	0.26 ± 0.02^{a}	0.53 ± 0.00^{b}	1.05 ± 0.07^{c}	0.26 ± 0.01^{a}	0.26 ± 0.00^{a}	0.53 ± 0.05^{b}	1.05 ± 0.01^{c}	0.26 ± 0.03^{a}
Streptomycin		**MIC**	0.09 ± 0.00^{a}	0.17 ± 0.02^{b}	0.17 ± 0.01^{b}	0.04 ± 0.00^{a}	0.17 ± 0.04^{b}	0.17 ± 0.00^{b}	0.26 ± 0.01^{c}	0.17 ± 0.00^{b}
		MBC	0.37 ± 0.02^{a}	0.37 ± 0.00^{a}	0.49 ± 0.03^{a}	0.37 ± 0.02^{a}	1.24 ± 0.00^{c}	0.49 ± 0.03^{a}	0.74 ± 0.07^{b}	0.49 ± 0.03^{a}

Indicated letters mean significant difference ($p < 0.05$)

78 **Table 7** Antifungal activities of *H. orphanidis* and standard by microdilution method in mg ml^{-1} (means ± SD)

Fungi/ *H. orphanidis* Es/Standard			*C. albicans*	*T. viride*	*P. ochrochloron*	*P. funiculosum*	*A. fumigatus*	*A. versicolor*	*A. ochraceus*	*A. niger*
ME	Aerial parts	**MIC**	6 ± 0.02^{b}	6 ± 0.07^{b}	6 ± 0.02^{b}	6 ± 0.00^{b}	6 ± 0.01^{b}	3 ± 0.02^{a}	6 ± 0.02^{b}	6 ± 0.05^{b}
		MFC	14 ± 0.03^{b}	8 ± 0.01^{a}	8 ± 0.10^{a}	8 ± 0.03^{a}	8 ± 0.01^{a}	8 ± 0.10^{a}	8 ± 0.02^{a}	18 ± 0.05^{c}
	Root	**MIC**	4 ± 0.03^{c}	3 ± 0.02^{b}	2 ± 0.03^{a}	2 ± 0.01^{a}	3 ± 0.05^{b}	2 ± 0.01^{a}	2 ± 0.01^{a}	3 ± 0.07^{b}
		MFC	14 ± 0.01^{b}	4 ± 0.00^{a}	4 ± 0.05^{a}	4 ± 0.01^{a}	4 ± 0.01^{a}	3 ± 0.00^{a}	3 ± 0.00^{a}	14 ± 0.03^{b}
EE	Aerial parts	**MIC**	6 ± 0.05^{c}	4 ± 0.01^{b}	4 ± 0.00^{b}	2 ± 0.05^{a}	4 ± 0.0^{b}	2 ± 0.00^{a}	4 ± 0.01^{b}	4 ± 0.03^{b}
		MFC	8 ± 0.02^{c}	6 ± 0.00^{b}	5 ± 0.01^{a}	5 ± 0.07^{a}	5 ± 0.10^{a}	4 ± 0.05^{a}	6 ± 0.07^{b}	18 ± 0.10^{d}
	Root	**MIC**	8 ± 0.00^{b}	8 ± 0.01^{b}	4 ± 0.00^{a}	8 ± 0.01^{b}	4 ± 0.02^{a}	4 ± 0.02^{a}	8 ± 0.02^{b}	10 ± 0.01^{c}
		MFC	14 ± 0.02^{c}	10 ± 0.01^{b}	8 ± 0.02^{a}	10 ± 0.00^{b}	8 ± 0.01^{a}	8 ± 0.03^{a}	10 ± 0.05^{b}	14 ± 0.02^{c}
AE	Aerial parts	**MIC**	10 ± 0.01^{a}	10 ± 0.07^{a}	12 ± 0.07^{b}	10 ± 0.03^{a}	10 ± 0.03^{a}	12 ± 0.03^{b}	10 ± 0.05^{a}	14 ± 0.03^{c}
		MFC	18 ± 0.08^{b}	14 ± 0.03^{a}	16 ± 0.03^{b}	12 ± 0.01^{a}	18 ± 0.05^{b}	16 ± 0.1^{b}	16 ± 0.02^{b}	$> 18 \pm 0.07^{c}$
	Roots	**MIC**	10 ± 0.01^{b}	12 ± 0.03^{c}	10 ± 0.02^{b}	10 ± 0.02^{b}	10 ± 0.03^{b}	5 ± 0.01^{a}	10 ± 0.02^{b}	10 ± 0.00^{b}
		MFC	12 ± 0.01^{a}	14 ± 0.07^{b}	12 ± 0.03^{a}	12 ± 0.1^{a}	14 ± 0.02^{b}	10 ± 0.03^{a}	14 ± 0.0^{b}	$> 18 \pm 0.01^{c}$
Fluconazole		**MIC**	0.02 ± 0.01^{a}	1 ± 0.01^{c}	1.0 ± 0.07^{c}	0.25 ± 0.0^{a}	0.5 ± 0.02^{b}	0.13 ± 0.02^{a}	0.5 ± 0.0^{b}	0.25 ± 0.03^{a}
		MFC	0.03 ± 0.00^{a}	1.5 ± 0.03^{d}	1.5 ± 0.03^{d}	0.5 ± 0.05^{b}	1 ± 0.02^{a}	0.5 ± 0.03^{b}	1 ± 0.05^{c}	1 ± 0.01^{c}

79 Indicated letters mean significant difference ($p < 0.05$)

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Table 8 Effects of *H. orphanidis* on biofilm formation of *P. aeruginosa* PAO1 (%)

H. orphanidis Es/EO/Standards		Biofilm formation*		
		0.5MIC (% ± SE)	**0.25MIC (% ± SE)**	**0.125MIC (% ± SE)**
ME	Aerial parts	70.53 ± 0.50	65.94 ± 1.53	66.91 ± 1.58
	Roots	77.78 ± 2.34	68.84 ± 2.47	66.67 ± 1.82
EE	Aerial parts	67.87 ± 1.65	66.43 ± 2.73	52.66 ± 2.71
	Roots	69.08 ± 0.87	72.22 ± 1.27	77.05 ± 0.78
AE	Aerial parts	74.64 ± 1.24	69.32 ± 1.48	63.53 ± 1.53
	Roots	74.88 ± 2.65	75.12 ± 0.65	75.36 ± 2.43
EO	Aerial parts	81.64 ± 2.73	80.92 ± 2.05	81.16 ± 2.73
Ampicillin		69.16 ± 0.65	56.46 ± 0.46	92.16 ± 0.37
Streptomycin		49.40 ± 0.46	70.97 ± 0.36	88.36 ± 0.42

*Biofilm formation values were calculated as: ((mean A_{620} control well)/(mean A_{620} treated well)/mean A_{620} control well) x 100.

Values are expressed as means ± SE.

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103 **Table 9** Effects of *H. orphanidis* on twitching and flagella mobility of *P. aeruginosa* PAO1

Parameters/ *H. orphanidis* Es/EO/Standards		Colony diameter (mm ± SE)	Flagella diameter (µm)	Colony colour	Colony edge
ME	Aerial parts	18.00 ± 3.00	56 - 104	White	Regular flagella
	Roots	12.33 ± 1.15	40 - 64	White	Reduced flagella
EE	Aerial parts	20.33 ± 4.51	32 - 64	Light green	Tiny flagella
	Roots	16.33 ± 3.21	24 - 56	Light green	Regular flagella
AE	Aerial parts	15.00 ± 1.00	32 - 64	White	Regular flagella
	Roots	11.33 ± 1.15	24 - 40	White	Reduced flagella
EO		10.33 ± 0.58	32 - 48	Light green	Reduced flagella
Streptomycin		11.00 ± 1.00	24 - 56	Green	Tiny flagella
Ampicillin		13.33 ± 5.03	16 - 56	Green	Regular flagella
Control (10^9 CFU ml^{-1})		12.00 ± 1.00	56 - 80	Light green	Regular flagella

104 Values for colony diameter are expressed as means ± SE.

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