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Anti-quorum sensing activity, toxicity in zebrafish (Danio rerio) embryos and phytochemical characterization of *Trapa natans* leaf extracts

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Abstract

Ethnopharmacological relevance: Trapa natans L. (water chestnut or water caltrop) is a widespread aquatic plant, which has been cultivated for food and traditional medicine since ancient times. Pharmacological studies showed that water chestnut exhibits the wide range of biological activities, such as antimicrobial, antioxidative, analgesic, anti-inflammatory, as well as antiulcer.

Aim of the study: Evaluation of anti-virulence potential and toxicity of *T. natans* methanol (TnM), acetone (TnA) and ethyl acetate (TnEA) leaf extracts.

Materials and methods: The anti-quorum sensing activity of Tn extracts was addressed by measuring their effects on biofilm formation, swarming motility and pyocyanin and elastase production in *Pseudomonas aeruginosa*. Specific *P. aeruginosa* biosensors were used to identify which of the signaling pathways were affected. The lethal and developmental toxicity of extracts were addressed *in vivo* using the zebrafish (*Danio rerio*) model system. The phenolic composition of *T. natans* leafs extracts was analyzed by a linear ion trap-OrbiTrap hybrid mass spectrometer (LTQ OrbiTrapMS) and UHPLC system configured with a diode array detector (DAD) hyphenated with the triple quadrupole mass spectrometer.

Results: Subinhibitory concentrations of Tn leaf extracts (0.2 MIC) inhibited pyocyanin and elastase production up to 50% and 60%, respectively, and reduced swarming zones, comparing to non-treated *P. aeruginosa*. TnA inhibited biofilm formation by 15%, TnM showed a stimulatory effect on biofilm formation up to 20%, while TnEA showed no effect. The bioactive concentrations of TnM and TnA were not toxic in the zebrafish model system. Twenty-two phenolic compounds were tentatively identified in TnM, where thirteen of them were identified in *T. natans* for the first time. Tn extracts, as well as their major components, ellagic and ferulic acids, demonstrated the ability to interfere with *P. aeruginosa* Las and PQS signaling pathways.

Conclusions: This study demonstrates anti-virulence potential of *Tn* leaf extracts against medically important pathogen *P. aeruginosa* and confirms the ethnopharmacological application of this plant against microbial infections.

Keywords: *Trapa natans*; *Pseudomonas aeruginosa*; quorum sensing; toxicity; zebrafish

1. Introduction

Trapa natans, commonly named the water chestnut or water caltrop, is a widespread floating-leaved aquatic plant belonging to genus *Trapa*, family Trapaceae (Radojević et al., 2016). Water chestnut is found in temperate and tropical freshwater wetlands, rivers, lakes, ponds, and estuaries. The plant is native to Southern Europe, Asia, and Africa and has been introduced in North America and Australia. The herbs have two types of leaves, submerged leaves which are finely divided feather-like, born along the length of the stem, and undivided floating leaves born in a rosette at the water's surface. The floating leaves are rhomboid, fan-shaped and have toothed edges. The cord-like stems are spongy and buoyant. The stems are anchored to the bottom with numerous branched roots. The plants bloom from June to September giving white solitary flowers that open above the water surface in the afternoons. The fruit is a nut, which depending on the variety can be two- or four horned (Hummel and Kiviat, 2004).

Water chestnuts have been cultivated for food and traditional medicine in China and India since ancient times. The fruits are the rich source of diverse nutrients including starch, proteins, and minerals (Chiang et al., 2009). Their good pharmacological properties such as antipyretic, diuretic, hemostatic, constipating, antidiarrheal, have been recognized and applied in traditional Chinese and Indian medicine for centuries (Adkar et al., 2014). The numerous

biological activities of different *T. natans* extracts have been demonstrated *in vitro* and *in vivo* (Zhu, 2016) and include anticancer (Lin et al., 2013), anti-inflammatory (Kim et al., 2015), anti-hyperglycemic (Kharbanda et al., 2014; Huang et al., 2016), anti-hepatotoxic (Kharbanda et al., 2014), antioxidant (Malviya et al., 2010), or antimicrobial (Radojević et al., 2016). Most of the bioactivities previously reported came from the peel and kernel extracts, while the leaves extracts have been much less analyzed.

Water chestnut is a rich source of carbohydrates, proteins, lipids, essential minerals as well as vitamins (B1, B2, B5, B6, E, A, C). Also, water chestnut contains dietary fibers and polyphenols such as phenolic acids and flavonoids as well as hydrolyzable tannins, which are bioactive compounds believed to be responsible for antidiabetic, antioxidant, antimicrobial and anticancer activities (Huang et al., 2016). Peels of water chestnuts tend to have much higher content of phenolic compounds than kernels (Malviya et al., 2010). The phenolic composition of ethanol extracts of *T. natans* roots/leaves/pulp/hulls has been analyzed by HPLC-DAD and nuclear magnetic resonance (NMR) (Zhu, 2016). Phenolic acids and flavonoids of seeds of green-, red-, and black-colored *T. natans* were analyzed by high-performance thin-layer chromatography (HPTLC) (Niranjan et al., 2013). In some of the above-mentioned studies, the plant part used for extraction was untraceable (Zhu, 2016). To the best of our knowledge, comprehensive phenolic profiling of *T. natans* leaves has not been reported so far. In this study, the combination of ultrahigh-performance liquid chromatography (UHPLC) coupled with Orbitrap MS/MS mass analyzer and UHPLC system configured with a diode array detector (DAD) hyphenated with a triple quadrupole mass spectrometer was used for analysis of *T. natans* leaves extracts.

Moderate antibacterial and anti-biofilm activities that have previously been demonstrated for *T. natans* leaf extracts (Radojević et al., 2016) led us to examine their anti-virulence potential and the activity on bacterial communication. Bacterial cell-to-cell signaling, so-called quorum sensing (QS), is involved in the regulation of virulence factors production and biofilm formation, which are crucial for pathogenicity and colonization of eukaryotic hosts. QS relies on the production, accumulation, detection, and population-wide response to extracellular signaling molecules called autoinducers (AI) (Paczkowski et al., 2017). Among **G**ram-negative bacteria, QS is the best characterized in *Pseudomonas aeruginosa*, a major opportunistic pathogen and a common cause of hospital-acquired infections. *P. aeruginosa* has acquired resistance to commonly used antibiotics, therefore it is considered priority pathogen on the CDC ESKAPE pathogen list (Aloush et al., 2006; Lavilla Lerma et al., 2014). The QS system in *P. aeruginosa* is organized in a multi-layered hierarchy consisting of at least four interconnected signaling pathways: Las, Rhl, the PqsR-controlled quinolone system (PQS), and Integrated QS system (IQS) (Lee and Zhang, 2015). Three AI synthases LasI, RhlI, and PqsABCDH produce 3-oxo-C12-homoserine lactone (HSL), C4-HSL, and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. The three signaling pathways regulate the expression of many virulence factors, including pyocyanin, elastase, LasA protease, rhamnolipids, phospholipase, as well as biofilm formation (Papenfort and Bassler, 2016). Interference with QS signaling is expected to reduce or even block the virulence factors production leading to less severe infections of a level that can be cleared by the host's immune system. In addition, reducing biofilm formation may increase bacterial susceptibility to antibiotics, thus can improve the effectiveness of antibacterial therapies. Importantly, QS is not involved in bacterial growth, therefore inhibition of QS does not yield a strong selective pressure for development of resistance (Allen et al., 2014). The serious problem

of developing and spreading of antibiotic-resistant species has been faced worldwide, therefore this approach is considered a promising strategy to control bacterial infections alternative to conventionally used antibiotics (Njoroge and Sperandio, 2009).

The number of chemically synthesized compounds that inhibit bacterial communication rises, but they are mostly chemically modified QS antagonists, which have been discovered in plants extracts or essential oils (Koh et al., 2013; Silva et al., 2016). A lot of medicinal and food plants are important sources of compounds with anti-QS property against different bacterial species (Kordbacheh et al., 2017). Therefore, the previous research on antimicrobial properties of *T. natans* leaves extracts has been extended to their potential to inhibit QS-regulated virulence factors production in *P. aeruginosa.* In addition, the safety of the examined extracts for human usage has been addressed by evaluation of their toxicity *in vivo* in the zebrafish (*Danio rerio*) model system. Combination of two powerful and accurate mass spectrometry techniques was used for the first time to analyze *T. natans* leaves extracts. The interference of the extracts and their major components with specific QS signaling pathways has been addressed.

2. Materials and methods

2.1 Preparation of plant extracts

The floating leaves of plant *T. natans* were collected in Medjuvrsje Reservoir, Central Serbia, and the dried plant material was prepared as previously described (Radojević et al., 2016). Dried, ground plant material was extracted by maceration using acetone, methanol or ethyl acetate. Stock solutions of extracts (100 mg/mL, w/v) were prepared in DMSO (100%) and stored in dark at 4 °C. The highest concentration of extracts used was 1000 μg/mL.

2.2 Microbial strains and growth conditions

Pseudomonas aeruginosa PAO1 DSMZ 22644, *P. aeruginosa* PA14, *Chromobacterium violaceum* CV026 (McClean et al., 1997), PAOJP2/pKD-*rhlA (*Δ*rhlA* P*rhlA::lux*) (Duan and Surette, 2007), *P. aeruginosa* PA14-R3 (ΔlasI P*rsal*::*lux*) (Massai et al., 2011) and *P. aeruginosa* PAO1 Δ*pqsA* (CTX *lux*::*pqsA*) (Fletcher et al., 2007) were used in this study. Bacterial strains were grown in Luria Bertani (LB) broth at 37 °C on a rotary shaker at 180 rpm.

2.3 Antimicrobial susceptibility tests for planktonic cells

The minimum inhibitory concentrations (MIC) of *Trapa natans* extracts were determined according to standard broth microdilution assays recommended by the Clinical and Laboratory Standards Institute (M07-A9 document) (CLSI, 2012). The inoculums were 10^5 colony forming units (cfu)/mL. The MIC value corresponds to the lowest concentration that inhibits the growth after 24 h at 37 °C. DMSO was used for the growth control at the same concentration (up to 1%, v/v) as in the treatments. The assay was carried out in triplicate, and repeated two times.

2.4 Quorum sensing inhibition assays

2.4.1 Chromobacterium violaceum disk assay

The assay was performed as previously reported with some modifications (Borges et al., 2014). N-(hexanoyl)-l-homoserine lactone (HHL) (5 µM) was used as an inducer of violacein production in molten semi-solid LB agar (0.3% w/v). Cellulose disks containing extracts (500 µg/disk), Furanone C-30 (5 µg/disk; Sigma, Munich, Germany) or DMSO (5 µL) were placed on solidified agar and incubated for 24 h at 30 °C. Inhibition of violacein synthesis was defined by the presence of white haloes in a purple background.

2.4.2 Quantification of violacein production

C. violaceum CV026 was cultured overnight at 30 °C and 180 rpm in the presence of 5 μM HHL, Tn extracts or DMSO (0.1 or 0.5%, v/v) in LB broth. One ml from each culture was centrifuged at 13000 rpm for 10 min to precipitate insoluble violacein. The supernatant was discarded and 1 ml of DMSO was added to the pellet, vortexed vigorously until the violacein was completely solubilized, then centrifuged again at 13000 rpm for 10 min to remove the cells (Abudoleh and Mahasneh, 2017). The absorbance of violacein was read at 585 nm and the amount of pigment produced by bacteria grown in the presence of Tn extracts was compared to bacteria grown in the presence of DMSO. Experiments were performed in triplicate and repeated two times.

2.4.3 Static biofilm formation inhibition assay

Biofilm quantification assay was performed in 96-well microtiter plate format (Sarstedt, Germany) using a crystal violet (CV) staining of adherent cells (Merritt et al., 2005). An overnight culture of *P. aeruginosa* was diluted to 5×10^7 cfu/mL in LB containing either extracts, Furanone C-30 or DMSO (0.1%, v/v) and 100 μL was added to the wells. Biofilms were formed for 24 h and after washing adherent cells were stained with 0.1% (v/v) CV. Experiments were performed in hexaplicate and repeated three times.

2.4.4 Swarming assay

Swarm agar plates were made with M8 medium (Na_2HPO_4 (50 mM), KH_2PO_4 (25 mM), NaCl (4 mM)), supplemented with glucose (0.2%, w/v), casamino acid (0.5%, w/v), $MgSO_4$ (1 mM), and solidified with Bacto agar (0.5%, w/v) in the presence of extracts (100 μg/mL, w/v) or DMSO (0.1%, v/v). The plates were inoculated with bacterial culture (3 μL, OD_{600} = 0.4–0.6) (Ha et al., 2014) and incubated at 37 °C for 24 h. The experiment was repeated two times.

2.4.5 Pyocyanin assay

P. aeruginosa PA14 indicator strain was cultured overnight in the presence of extracts, Furanone C-30 or 0.1% DMSO, and pyocyanin in the supernatant was quantified using UV–vis spectrophotometer Ultrospec 3300pro (Amersham Biosciences, Piscataway, NJ, USA) at 695 nm as reported previously (O'Loughlin et al., 2013). Experiments were performed in triplicate and repeated two times.

2.4.6 Elastase assay

The elastolytic activity of the culture supernatants (incubated with extracts, Furanone C-30 or 0.1% DMSO) was determined using the Elastin Congo Red (ECR) method (Ohman et al., 1980). A 100 µl aliquot of the cell-free supernatant was added to 900 µl of ECR buffer containing 5 mg of ECR (Sigma, Munich, Germany). The mixture was incubated on a rotary shaker for 12 h at 37 °C. Insoluble ECR was removed and the absorption was measured at a wavelength of 485 nm. The elastase activity in the supernatant of treated cultures was presented relative to DMSO control (%). Experiments were performed in triplicate and repeated three times.

2.4.7 Interference of extracts and components with QS pathways

Overnight cultures of biosensors *P. aeruginosa* PA14-R3 (Δ*lasI* P*rsal::lux*), PAOJP2/pKD-*rhlA (*Δ*rhlA* P*rhlA::lux*) and *P. aeruginosa* PAO1 Δ*pqsA* (CTX *lux*::*pqsA*) were diluted to OD_{600} = 0.045, and incubated with 100 or 200 µg/mL *T. natans* extracts, 2.5 µg/mL Furanone C-30, or 100 µg/mL of their major components (ellagic, ferulic and gallic acids, all from Sigma, Munich, Germany, prepared as 100 mg/mL stock solutions in 100% DMSO) in the presence of specific autoinducers 3oxo-C12HSL, C4-HSL and HHQ, respectively. Cell density (OD_{600}) and bioluminescence (light counts per second, LCPS) were simultaneously measured after 4 h of incubation using Tecan Infinite200 multiplate-reader (Tecan Group Ltd., Switzerland).

Luminescence values were normalized per cell density. The assays were carried out in quadruplicate and repeated three times.

2.5 Cell culture

MRC5 human lung fibroblasts were obtained from the American Type Culture Collection (ATCC). Cells were maintained as monolayer cultures in RPMI-1640 supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin and 10% (v/v) FBS (all from Sigma, Munich, Germany). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.5.1 Cytotoxicity assay

Cytotoxicity on MRC5 cells was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Hansen et al., 1989). The assay was carried out after 48 h of cell incubation in the media containing extracts at concentrations ranging from 10 to 200 µg/mL. The results are presented as concentrations of extracts that inhibited 50% cell viability (IC_{50}).

2.6 In vivo toxicity evaluation

The *in vivo* toxicity assessment of *T. natans* extracts was carried out in the zebrafish *(Danio rerio*) model according to the OECD 2013 guidelines for the testing of chemicals (OECD, 2013). All experiments involving zebrafish were performed in compliance with the European directive 2010/63/EU and the ethical guidelines of the Guide for Care and Use of Laboratory Animals of the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade. The assay was performed according to the protocol previously described by Savic et al. with some modifications (Savic et al., 2016). Briefly, zebrafish embryos were produced by pair-wise mating

of wild-type adults, collected and distributed into 24-well plates containing 10 embryos per well in 1 mL embryos water (0.2 mg/mL of Instant Ocean® Salt in distilled water), and raised at 28 °C. For assessing lethal and developmental toxicity, embryos at the 6 hours post fertilization (hpf) stage were treated with eight concentrations of each extract (10, 25, 50, 100, 200, 300, 400, and 500 µg/mL). DMSO (0.25%) was used as a negative control. Experiments were performed three times using 30 embryos per concentration.

Apical endpoints used for the toxicity evaluation (Table S1) were recorded at 24, 48, 72, and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan). Dead embryos were counted and discarded every 24 h. At 96 hpf, embryos were inspected for heartbeat rate, anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed, and killed by freezing at -20 °C for ≥ 24 h.

The LC_{50} value (the concentration at which 50% of embryos were dead) was determined by the program ToxRatPro (ToxRat®, Software for the Statistical Analysis of Biotests, ToxRat Solution GmbH, Alsdorf, Germany, Version 2.10.05) using the probit analysis with linear maximum likelihood regression.

2.7 UHPLC- LTQ Orbitrap MS qualitative analysis

Chromatographic separation was conducted using an ultrahigh-performance liquid chromatography (UHPLC) system consisting of a quaternary Accela 600 pump and Accela autosampler (ThermoFisher Scientific, Bremen, Germany). The UHPLC system was coupled to a linear ion trap hybrid mass spectrometer (LTQ OrbiTrap MS) equipped with heated electrospray ionization probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). A Syncronis C18 column (100 × 2.1 mm, 1.7 mm particle size, Thermo Fisher Scientific**,** Bremen, Germany) was

used for separation of analyzed compounds. The MS analysis was performed in negative ion mode. For current analysis at a flow rate of 0.250 mL min^{-1} the mobile phase consisted of (A) water + 0.1% acetic acid and (B) acetonitrile. Injection volume was 5 μL and linear gradient programs were as follows: 0.0-1.0 min 5% B, 1.0-14.0 min from 5% to 95% (B), 14.0-14.1 min from 95% to 5% (B), and 5% (B) for 6 min. Parameters of the ion source were as follows: source voltage 4.5 kV, capillary voltage 4 V, tube lens voltage 59.11 V, capillary temperature 275 °C, sheath and auxiliary gas flow (N_2) 30 and 7 AU, respectively. The data were collected in the m/z range of *m/z* 100-1000. The resolution was set to 30,000 for full scan analysis. Each phenolic compound was identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. Xcalibur software (version 2.1) was used for the instrument control, data acquisition and data analysis (Ristivojevic et al., 2015).

2.8 UHPLC-MS/MS quantitative analysis

Quantification of ten phenolic compounds in extracts of *T. natans* was performed using Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific) configured with a diode array detector (DAD) and a triple quadrupole mass spectrometer (TSQ Quantum Access Max, ThermoFisher Scientific). An analytical Hypersil gold C18 column (50 × 2.1 mm) with 1.9 mm particle size (ThermoFisher Scientific) thermostated at 30 **°C** was used for separation. The mobile phase consisted of 0.2% acetic acid in water (A) and LC-MS grade acetonitrile (B), was applied with the flow rate of 0.4 mL/min in the gradient elution as previously described (Banjanac et al., 2017)**.** The injection volume was 10 μL. Settings of a triple quadrupole mass spectrometer (qqqMS), equipped with a heated electrospray ionization (HESI) source, were as follows:

vaporizer temperature 450 °C, spray voltage 4000 V, sheet gas (N_2) pressure 50 AU, ion sweep gas pressure 0 AU and auxiliary gas pressure at 20 AU, capillary temperature at 320 °C, skimmer offset 0 V. The mass spectrometer was operated in negative ion mode, while collision energy was 30 eV. Compounds were quantified by the external standard quantification procedure. Working standard solutions were prepared by dissolving the stock solution of a pure compound to obtain concentration 100 mg/mL, and other calibration levels were obtained by diluting the solutions with methanol. Working standard solutions were prepared at 15 levels, in the concentration range from 100 mg mL to 0.001 mg/mL, which was used for the determination of the limit of detection (LOD), limit of quantitation (LOQ), linearity, repeatability, and sensitivity of the developed UHPLC-qqqMS method. LOD and LOQ were determined as peak-to-peak values by the signal-to-noise ratios (S/N), with S/N > 3 and > 10 for LOD and LOQ, respectively. Six replicates of each compound were run for LOD and LOQ testing. Regressions were calculated for each of the calibration curves, and they all showed good linearity with correlation coefficients between r = 0.990 and 0.999, $p < 0.001$.

2.9 Statistical analysis

The results were analyzed by Student's t-test using SPSS version 20 software. A P-value lower than 0.05 was considered as statistically significant.

3. Results

3.1 Detection of anti-QS activity of T. natans extracts in C. violaceum

Before addressing the effects of *T. natans* methanol (TnM), acetone (TnA) and ethyl acetate (TnEA) extracts on bacterial QS, their antimicrobial activity was tested against three bacterial

strains used in this study (Table 1). The minimal inhibitory concentrations (MICs) of all extracts were 500 µg/mL or higher.

Table 1. Antibacterial activity of *T. natans* methanol (TnM), acetone (TnA) and ethyl acetate (TnEA) extracts determined after 24 h incubation.

	MIC [a] (µg/mL)		
Bacterial strain	TnM	TnA	TnEA
P. aeruginosa PAO1	500	500	500
P. aeruginosa PA14	500	500	1000
C. violaceum CV026	>1000	1000	>1000

[a] The minimum inhibitory concentration (MIC) determined as the lowest concentration of compound at which no evident growth was observed.

The interference of *T. natans* extracts with bacterial QS was addressed in acyl homoserine lactone (AHL)-based *in vitro* QS competition assay using *C. violaceum* CV026 as a test strain. *C. violaceum* produces water insoluble purple pigment violacein as a result of the interaction of its AHL receptor CviR with exogenously provided AHLs such as N-hexanoyl homoserine lactone (HHL). Binding of a specific inhibitor to the receptor inhibits violacein production. The zones of violacein inhibition in *C. violaceum* CV026 disk assay (12±1 mm and 10±1 mm for TnM and TnA, respectively) showed that one or more components in TnM and TnA could compete with HHL for CviR receptor (Figure 1a). TnEA also inhibited violacein production but to a smaller extent. Halogenated furanone (Furanone C-30) was used as positive control for QS inhibition (Martinelli et al., 2004). The production of violacein was quantified in planktonic *C. violaceum* CV026 cultures induced with HHL and grown in the presence of *T. natans* extracts (Figure 1b). All three extracts inhibited violacein production up to 50% when

applied at subinhibitory concentrations. These results suggested that *T. natans* extracts contain components that can interfere with AHL-regulated QS pathway.

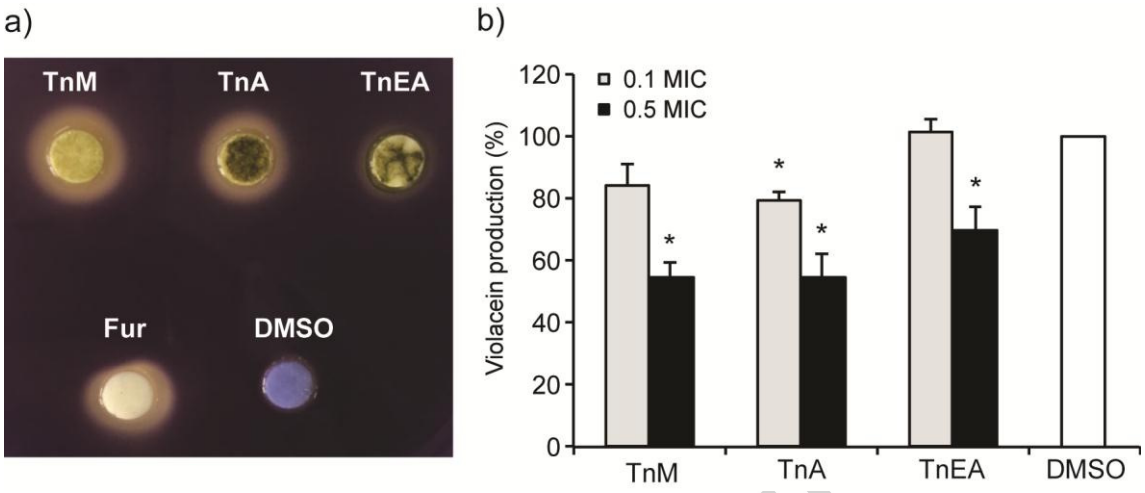

Figure 1: Anti-quorum sensing activity of *T. natans* extracts detected by *C. violaceum* CV026. (a) Effect of extracts (500 µg/disc) and Furanone C-30 (5 µg/disc) on violacein production detected by disk assay and (b) quantification of violacein production in liquid cultures in the presence of 100 µg/mL (0.1 MIC) or 500 µg/mL (0.5 MIC) extracts. Values are presented as mean ± SD. *P<0.05.

3.2 Effects of T. natans extracts on quorum sensing in P. aeruginosa

The recent report showed strong anti-biofilm formation activity of *T. natans* leaves extracts when applied in concentrations close to their MIC values (Radojević et al., 2016).

To address whether *T. natans* extracts could inhibit biofilm formation in *P. aerugonosa* PAO1 through interference with QS the effect was observed at subinhibitory concentrations of the extracts. TnA applied at concentrations 50-100 µg/mL (0.1 MIC- 0.2 MIC) showed 15%

reduction in biofilm formation (Figure 2). TnM stimulated the formation of biofilms up to 20%, while TnEA had no effect when applied in the same concentrations.

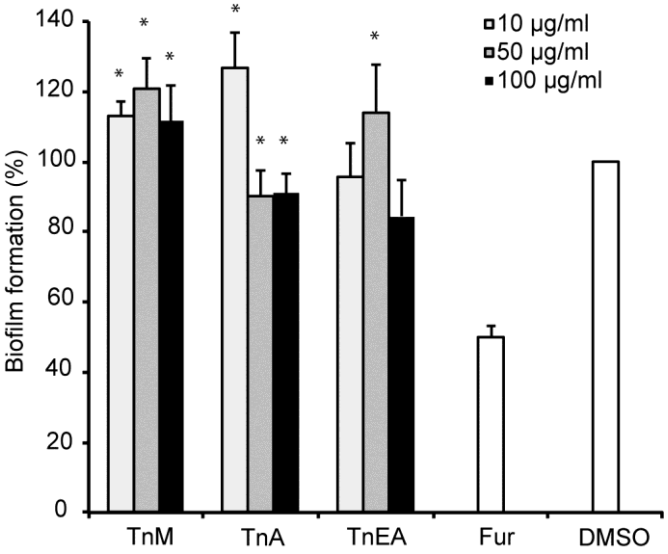

Figure 2. Effects of *T. natans* methanol (TnM), acetone (TnA), ethyl acetate (TnEA) extracts and Furanone C-30 (Fur; 25 µg/mL) on biofilm formation in *P. aeruginosa* PAO1. Values are presented as mean ± SD. *P<0.05.

Another *P. aeruginosa* feature regulated by QS is swarming, a multicellular type of motility which is associated with bacterial virulence and antibiotic resistance (Overhage et al., 2008). All three Tn extracts reduced *P. aeruginosa* swarming motility at 0.2 MIC with TnM showing the strongest swarming inhibitory activity (Figure 3).

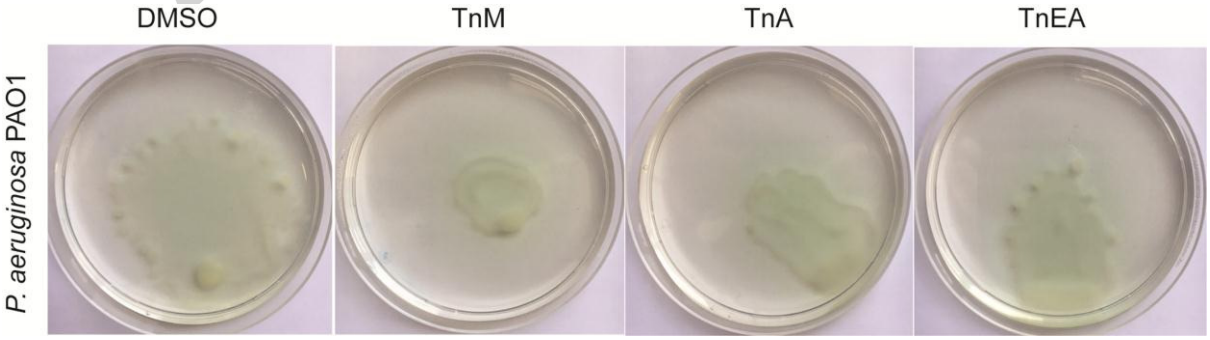

Figure 3. Effects of *T. natants* extracts (0.2 MIC) on swarming motility in *P. aeruginosa* PAO1. Pictures were taken 24 h after inoculation.

The interference of *T. natans* extracts with QS in *P. aeruginosa* was further examined by testing their effects on pyocyanin and elastase synthesis (Figure 4). TnM and TnA dose-dependently reduced the amount of pyocyanin in the culture supernatant between 25 and 40%, respectively (Figure 4a). In the presence of 100 µg/mL TnEA the pyocyanin production was inhibited by 50%, while with the increase of TnEA concentration the inhibitory effect on pyocyanine production was lost. All extracts inhibited elastase production in *P. aeruginosa* supernatants between 60 and 65%, or 60 and 80% when used in concentration 0.2 MIC or 0.4 MIC, respectively (Figure 4b**).** The strongest inhibition of elastase activity (up to 80%) was observed in the presence of 0.4 MIC TnEA. Furanone C-30 showed strong inhibition of pyocyanin production at 5 µg/mL, while the same concentration of the compound exhibited no effect on elastase production. The increase of Furanone C-30 concentration up to 25 µg/mL showed no effect on elastase activity (data not shown).

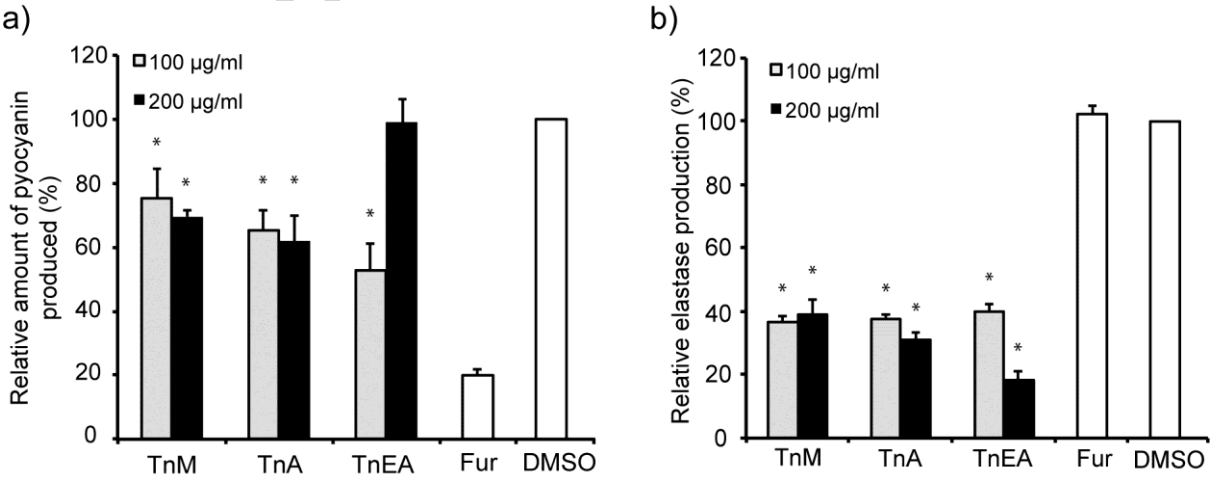

Figure 4. (a) Inhibition of pyocyanin and (b) elastase production in the presence of *T. natants* extracts or Furanone C-30 (5 μg/mL). Values are presented as mean ± SD. *P<0.05.

Taken together, all these results demonstrate that *T. natans* leaf extracts can modulate virulence factors production in *P. aeruginosa* by interfering with QS signaling.

3.3 Analysis of T. natans extracts toxicity

To address whether anti-QS active concentrations of *T. natans* leaves extracts are safe for human usage, *in vitro* toxicity to human lung fibroblasts (MRC5) was tested first. All extracts showed 50% cytotoxicity in concentrations 40-50 μg/mL upon 48 h treatments (Table 2).

Table 2. Cytotoxicity of *T. natans* extracts to human lung fibroblast (MRC5) cell line after 48 h exposure at a range of concentrations.

Extract	IC_{50} [a] (μg/mL)
TnM	50
TnA	40
TnEA	50

[a] IC_{50} concentration is determined as the concentration of extract at which 50% of cells was killed.

The toxicity of *T. natans* extracts has been further evaluated *in vivo* using the zebrafish (*Danio rerio*) model. Zebrafish embryos were treated very early in embryonic development (at 6 hpf), and inspected over a period of 120 h for the survival and an appearance of teratogenic malformations. Obtained results revealed that TnM was the least toxic, while TnEA appeared the most toxic for the embryos. According to the determined LC_{50} values (Figure 5) extracts were ranked by their toxicity: TnM < TnA < TnEA. Namely, embryos exposed to TnM showed no

signs of toxicity (developmental and cardiovascular toxicity) at any tested concentrations up to 500 µg/mL. TnA was not toxic in zebrafish embryos at the doses up to 100 µg/mL, while at 200 µg/mL embryos were inhibited for the hatching, and some of them (30%) showed cardiovascular abnormalities. None of the unhatched embryos died up to 120 hpf. On the other side, the embryos exposed to TnEA in the doses ≥ 50 µg/mL exhibited signs of cardiotoxicity, while all embryos at 200 µg/mL were unhatched and died within a period of 96 - 120 hpf. The DMSO-treated embryos (negative control) hatched until 96 h.

Taken together, *in vivo* toxicity evaluation revealed the biosafety of TnM at concentrations with anti-QS activity.

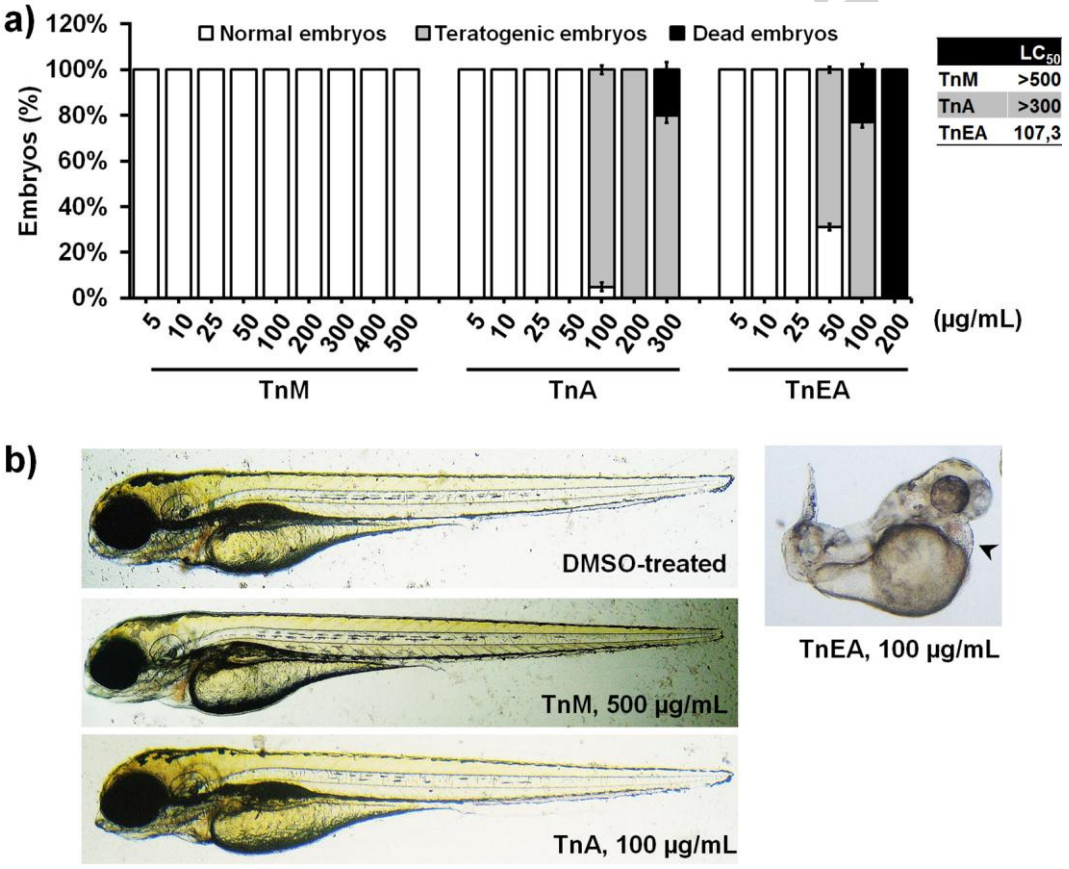

Figure 5. (a) Toxicity assay on zebrafish embryos exposed to different concentrations of different extracts of *T. natans* expressed as the LC_{50} values (µg/mL). (b) Morphology of the

zebrafish embryos at 96 hpf exposed to 500 µg/mL of TnM (normal embryos), 100 µg/mL of TnA (normal embryo), and 100 µg/mL of TnEA (manually dechorionated dead and teratogenic embryo with pericardial edema). Pericardial edema was denoted with an arrowhead.

3.4 Chemical composition of T. natans leaf extracts

3.4.1 Identification of phenolic compounds

Methanol extract, as the least toxic among studied ones, was chosen for phytochemical analysis and identification of the constituents contributing to the observed anti-QS activity. For that purpose, the UHPLC-LTQ OrbiTrap-MS system was used as a powerful tool for phenolic profiling.

Twenty-two phenolic compounds comprising the *p*-hydroxybenzoic acid, ten phenolic acids and derivatives, four flavonoids, and seven flavonoid glycosides, were tentatively identified in TnM (Table 3). Compounds were identified based on comparison of their retention times [t_R, (min)], molecular formula, calculated and accurate masses [$[M-H]^-$, (*m/z*)], mass accuracy errors [Δ, (mDa)], characteristic MS^2 and MS^3 fragment ions, with corresponding data obtained with internal standards and from the literature. Base peak chromatogram of TnM is presented in the Supporting material (Figure 1S).

From twenty-two identified phenolic compounds, nineteen compounds were detected for the first time in *T. natans* leaves extract. To the best of our knowledge, only three phenolic acids – caffeic 7, *p*-coumaric 8, and ferulic 11 acids, were found in *T. natans* leaf extracts up today (Djurdjevic, 2000; Stoicescu et al., 2012; Zhu, 2016). Moreover, thirteen substances, compounds **1**, **2**, **6**, **10**, **13**, **15 – 22**, were identified for the first time in *T. natans*, while the nine compounds listed in Table 3 were found previously in the corresponding extracts of the other parts of *T.*

natans, such as roots, pulp, hulls, fruits and pericarps, and peels (Stoicescu et al., 2012; Niranjan et al., 2013; Kharbanda et al., 2014; Huang et al., 2016; Zhu, 2016).

Phenolic acids were identified either solely or as corresponding glycosides. Derivatives **1** and **2**, two structural isomers, were identified as gallic acid hexosides based on $[M - H]^-$ ion at 331.0670 *m/z* and characteristic MS^2 and MS^3 fragments at 169 *m/z* and 125 *m/z*, respectively (Frohlich et al., 2002; Pavlovic et al., 2016). Compound **10** was assigned as one of the reported structural isomers of *p*-coumaroyl-di-galloyl-*O*-glucose (Wang et al., 2013) based on molecular ion $[M - H]^-$ at 629.11426 *m/z*, and characteristic MS^2 fragments at 459 *m/z* and 465 *m/z* and also MS^3 fragment ions at 313 *m/z* and 294 *m/z* (Wang et al., 2013).

Quantification of ten phenolic compounds in *T. natans* leaf extracts (Table 2S) showed that three phenolic acids, namely gallic, ellagic, and ferulic, as well as quercetin 3-*O*-galactoside (hyperoside), were present in a higher amounts, comparing to other compounds. The presence of caffeic, ferulic, and *p*-coumaric acids in *T. natans* leaf extract has been reported previously (Djurdjevic, 2000; Stoicescu et al., 2012). Additionally, ellagic and *p*-coumaric acids were detected in *T. natans* methanol extracts of pericarp powders (Yu and Shen, 2015).

This study is also the first report of the presence of four flavonoids **12**-**15** in *T. natans* leaf extracts. They showed retro-Diels–Alder (RDA) as characteristic fragmentation pathway of flavonoids (de Rijke et al., 2006). From identified flavonoids, two were previously reported in *T. natans* samples: quercetin (**13)** in fruits (Niranjan et al., 2013) and naringenin (**14)** in pericarps (Huang et al., 2016). Structural isomers **18** and **19** noticed at different t_R, were identified as kaempferol di-*O*-sophorosides based on $[M- H]^-$ ion at 609.1461 *m/z* and corresponding MS^2 fragments (Pavlovic et al., 2016).

Table 3. Polyphenolic compounds identified in the *T. natans* leaf methanol extract using UHPLC-LTQ OrbiTrap MS^n analysis in negative ion mode.

Peak [a]	t_R, (*min*)	Molecular formula $[M-H]^-$	Calculate mass, $[M-H]^-$ (*m/z*)	Accurate mass, $[M-H]^-$ (*m/z*)	Δ (mDa)	Fragmentation MS^n (*m/z*)	Identification [b]	Reference
Phenolic acids and their derivatives								
1	1.24	$C_{13}H_{15}O_{10}^-$	331.0670	331.0661	0.91	MS^2: 295(10), 169 (100), 150 (10); MS^3: 125(100);	Gallic acid hexoside isomer 1	(Frohlich et al., 2002; Pavlovic et al., 2016).
2	1.80	$C_{13}H_{15}O_{10}^-$	331.0670	331.0662	0.85	MS^2: 169(100); MS^3: 125(100);	Gallic acid hexoside isomer 2	(Frohlich et al., 2002; Pavlovic et al., 2016)
3	2.47	$C_7H_5O_5^-$	169.0147	169.0132	1.50	MS^2: 125 (100);	Gallic acid [c]	(Ristivojevic et al., 2015)
4	4.32	$C_7H_5O_4^-$	153.0197	153.0184	1.30	MS^2: 109 (100); MS^3: 81(60);	Protocatechuic acid [c]	(Ristivojevic et al., 2015)
5	5.18	$C_7H_5O_3^-$	137.0244	137.0239	0.52	MS^2: 109(10), 93 (100);	*p*-Hydroxybenzoic acid	(Ristivojevic et al., 2015)
6	5.29	$C_{15}H_{17}O_8^-$	325.0912	325.0912	0.00	MS^2: 265(20), 187(60), 163(90), 145(100), 119(10); MS^3: 117(100);	*p*-Coumaric acid hexoside	(Bystrom et al., 2008; Wang et al., 2013)
7	5.71	$C_9H_7O_4^-$	179.0350	179.0345	0.50	MS^2: 135 (50); MS^3: 91(100);	Caffeic acid [c]	(Vallverdu-Queralt et al., 2012; Ristivojevic et al., 2015)

8	6.61	$C_9H_7O_3^-$	163.0401	163.0394	0.70	MS^2: 119 (100);	*p*-Coumaric acid [c]	(Ristivojevic et al., 2015)
9	6.66	$C_{14}H_5O_8^-$	300.9989	300.9980	0.94	MS^2: 257(100); MS^3: 229(100);	Ellagic acid [c]	(Pavlovic et al., 2016)
10	6.87	$C_{29}H_{25}O_{16}$	629.1142	629.1127	1.53	MS^2: 459(20), 465(100); MS^3: 313(100), 295(20);	*p*-Coumaroyl-di-galloyl-*O*-glucose	(Wang et al., 2013)
11	9.10	$C_{10}H_9O_4^-$	193.0506	193.0496	1.00	MS^2: 178(70), 149(100); MS^3: 134(100);	Ferulic acid [c]	(Ristivojevic et al., 2015)
Flavonoids								
12	8.68	$C_{15}H_9O_7^-$	301.0354	301.0331	2.30	MS^2: 283(15), 271(60), 257(25), 179(100), 151(80); MS^3: 151(100);	Quercetin	(Ristivojevic et al., 2015)
13	9.38	$C_{15}H_{11}O_5^-$	271.0612	271.0603	3.32	MS^2: 253(100), 225(29); MS^3: 197(100);	Pinobanksin	(Pellati et al., 2011)
14	9.41	$C_{15}H_{11}O_5^-$	271.0612	271.0599	4.65	MS^2: 151(100); MS^3: 107(100);	Naringenin [c]	(Ristivojevic et al., 2015)
15	9.84	$C_{16}H_{11}O_7^-$	315.05103	315.0494	1.63	MS^2: 301(20), 300(100); MS^3: 283(40), 271(80), 255(30), 227(30), 151(100);	Rhamnetin	(Pellati et al., 2011)
Flavonoid Glycosides								
16	3.31	$C_{21}H_{21}O_{10}^-$	433.1140	433.1133	0.72	MS^2: 271(100), 272(15); MS^3: 151(100), 119(5), 107(40), 177(30);	Naringenin-7-*O*-hexoside	(Sanchez-Rabaneda et al., 2003; Vallverdu-Queralt et al., 2012; Mudric et al., 2017)
17	5.40	$C_{27}H_{29}O_{16}^-$	609.1461	609.1442	3.14	MS^2: 429(80), 327(10), 309(10), 285(100), 284(70), 255(20); MS^3: 257(100), 241(50), 229(40), 213(30), 151(70);	Kaempferol di-*O*-hexoside isomer 1	(Pavlovic et al., 2016)
18	6.19	$C_{27}H_{29}O_{16}^-$	609.1461	609.1437	3.96	MS^2: 447(10), 429(80), 285(100), 284(70), 255(20); MS^3: 257(100), 241(50), 229(40), 213(30), 151(70);	Kaempferol di-*O*-hexoside isomer 1	(Pavlovic et al., 2016)
19	6.19	$C_{27}H_{29}O_{16}^-$	609.1461	609.1443	1.80	MS^2: 301(100); MS^3: 151(100);	Rutin [c]	(Ristivojevic et al.,

								2015)
20	7.04	$C_{21}H_{19}O_{11}^-$	447.0932	447.0917	1.58	MS^2: 327(20), 285(80), 284(100), 255(10); MS^3: 255(100), 227(10);	Kaempferol-3-*O*-glucoside (Astragalin) [c]	(Sanchez-Rabaneda et al., 2003)
21	7.11	$C_{21}H_{19}O_{11}^-$	447.0932	447.0911	2.16	MS^2: 301(100), 300(35), 284(20); MS^3: 273(25), 257(20), 179(100), 151(75);	Quercetin 3-*O*-rhamnoside	(Falcao et al., 2013) (Sanchez-Rabaneda et al., 2003)
22	6.68	$C_{21}H_{19}O_{12}^-$	463.0882	463.0868	1.35	MS^2: 301(100), 300(30); MS^3: 273(25), 257(20), 179(100), 151(75);	Quercetin 3-*O*-galactoside [c]	(Sanchez-Rabaneda et al., 2003)

[a] Peak numbers corresponding to Fig. 1S. [b] Compounds were identified based on data presented in this table and comparison with corresponding data given in thereferences, if otherwise is not additionally specified. [c] Confirmed by available standards.

3.5 Identification of QS signaling pathways affected by T. natans extracts

Finally, the interference of TnM and TnA components with *P. aeruginosa* QS receptors was analyzed. The receptors' activity was quantified using three biosensors - *P. aeruginosa* PA14-R3 used to detect interaction with LasR, *P. aeruginosa* PAOJP2 used for measurements of RhlR activity**,** and *P. aeruginosa* PAO1 Δ*pqsA* used for detection of PqsR activity. Both TnM and TnA applied at a subinhibitory concentration (100 µg/mL) showed the presence of molecules that can interfere with Las pathway inhibiting LasR activity between 20 and 45% (Figure 6). In addition, both extracts stimulated Rhl and PQS pathways when applied in that concentration. The increase of extracts concentration up to 200 µg/mL did not affect their activities on Las and Rhl signaling. However, 200 µg/mL of TnM or TnA inhibited PQS signaling by 40% and 60%, respectively.

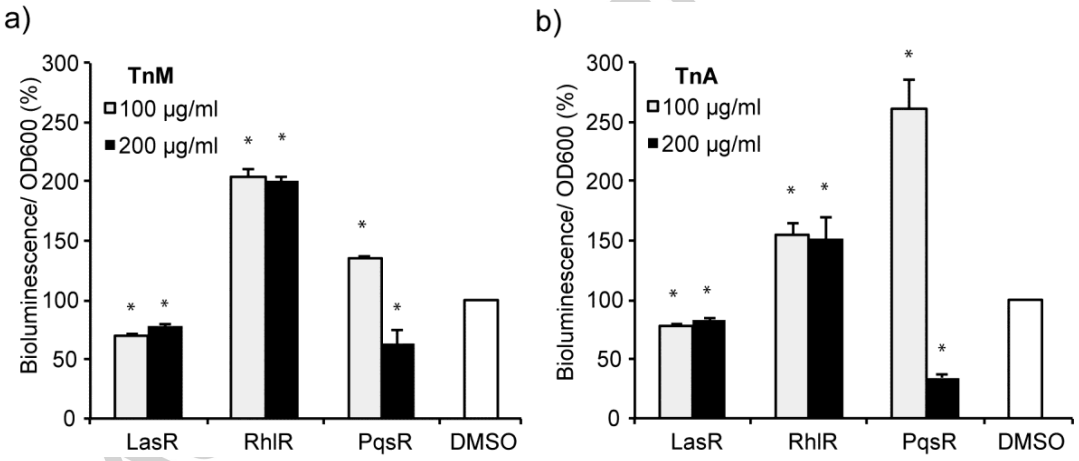

Figure 6. Effects of (a) methanol and (b) acetone *T. natants* extracts on the activity of QS receptors detected by bioluminescence measurements. Values are presented as mean ± SD. *P<0.05.

These data showed that compounds present in *T. natans* leaf extracts exhibited dose-dependent selectivity toward QS signaling pathways. TnM and TnA contain at least one component with Las antagonistic activity thus inhibiting long chain AHL-regulated signaling.

The higher concentration of the TnM and TnA (200 µg/mL) also showed PQS inhibitory activity suggesting that the compounds in extracts are weaker PqsR antagonists.

Ellagic, ferulic, and galic acids as the most abundant phenolic compounds identified in TnM and TnA, have also been analyzed for the ability to interfere with specific QS signaling pathways at non-bactericidal concentrations (Figure 7a and b). Ellagic and ferulic acids at 100 µg/mL inhibited LasR and PqsR activities by 35-40% and 30-40%, respectively. Ellagic acid stimulated RhlR activity by 25%, while ferulic acid inhibited Rhl signaling by 10%. Gallic acid stimulated Pqs signaling by 35%, but showed no effect on LasR and RhlR activities.

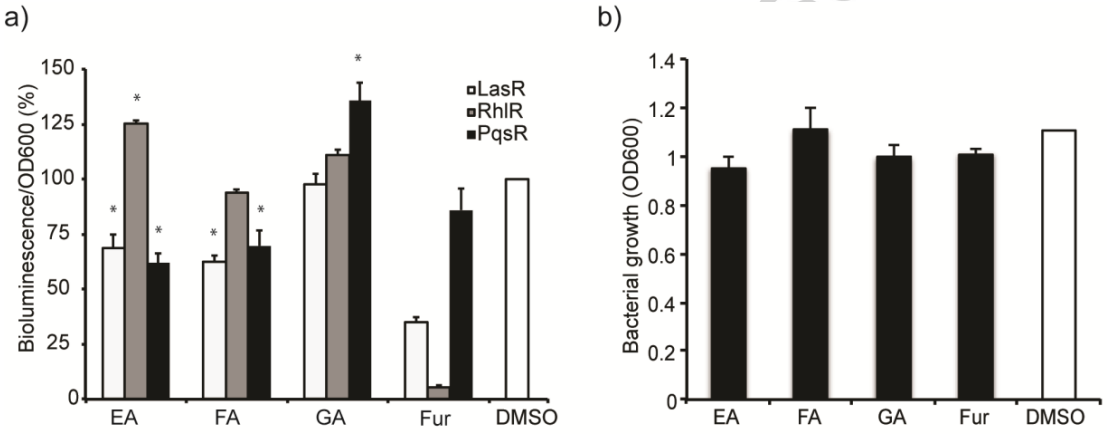

Figure 7. Effects of ellagic (EA), ferulic (FA), gallic (GA) acids (100 µg/mL) and Furanone C-30 (2.5 µg/mL) on **(a)** QS signaling detected by bioluminescence measurements and **(b)** the growth of *P. aeruginosa* PAO1. Values are presented as mean ± SD. *P<0.05.

4. Discussion

Trapa natans is an annual aquatic fruit plant found in tropical, sub-tropical and temperate zones of the world. Different parts of the plant have been used for food or traditional medicine (Karg, 2006; Vandana et al., 2015; Zhu, 2016). Antibacterial properties of different *T. natans* extracts have been studied by several groups demonstrating their activity against

Gram-positive and **G**ram-negative bacteria with MIC values ranging from 625 to >5000 µg/mL (Razvy et al., 2011; Yu and Shen, 2015; Radojević et al., 2016). Our results show that different leaf extracts, namely methanol (TnM), acetone (TnA) and ethyl acetate (TnEA), exhibit antibacterial activity in the concentrations from 500 to 1000 µg/mL, depending on bacterial species (Table 1). One of the promising strategies to fight bacterial infections and avoid development of resistant species is interference with cell-to-cell signaling (Bhardwaj et al., 2013; Chatterjee et al., 2016). Conventional anti-bacterial treatments with antibiotics affect bacterial survival and thus cause the development of the resistance. Interference with bacterial quorum sensing (QS) inhibits production of virulence factors and enables the host immune system to fight and resolve the infection in the absence of a selective pressure on the pathogen. In this study, different biosensor strains were used to evaluate *T. natans* leaf extracts as inhibitors of QS signaling. First, *C. violaceum* CV026 was used to detect interference with AHL-regulated signaling and showed that TnM, TnA, and TnEA inhibited QS-regulated violacein production when applied at concentration 0.5 MIC (Fig. 1). These results suggested that *T. natans* leaf extracts contain one or more compounds with AHL antagonistic activity. The anti-QS activity of *T. natans* extracts was further evaluated against clinically relevant pathogen *P. aeruginosa,* which regulates virulence factor production such as elastase, pyocyanin, proteases, rhamnolipids, or biofilm formation through QS signaling (Chatterjee et al., 2016). Biofilm formation is the major virulence determinant of *P. aeruginosa* and many other pathogenic bacteria, and is involved in low sensitivity or resistance to antibiotics (Bryers, 2008). A recent study (Radojević et al., 2016) demonstrated that *T. natans* leaf extracts such as ethanol, acetone, and ethyl acetate extracts inhibit biofilm formation in *P. aeruginosa* with BIC90 (the concentration that inhibits 90% of biofilm formation) ranging from 700 to 4000 µg/mL. These BIC90 concentrations were close to MIC values, therefore the observed activity was most likely caused by inhibition of bacterial growth. This study addressed a potential of TnM, TnA and TnEA leaf extracts to inhibit the

formation of biofilms at subinhibitory concentrations. Only TnA showed inhibitory activity on biofilm formation at 0.1 MIC and 0.2 MIC (Fig. 2), while TnM or TnEA displayed no effect or slightly stimulated biofilm formation at tested concentrations. Biofilm formation is closely associated with swarming motility, a QS-regulated virulence characteristic responsible for surface attachment and movements across semisolid surfaces. *T. natans* leaf extracts reduced swarming motility of *P. aeruginosa*, with the strongest inhibition observed for TnM, and the lowest effect observed for TnEA (Fig. 3).

Other virulence factors required for establishing full virulence in *P. aeruginosa* infections are pyocyanin and elastases. Pyocyanin is a blue redox-active pigment that promotes virulence by interfering with several cellular functions in host cells including electron transport, cellular respiration, energy metabolism, gene expression, and innate immune mechanisms (Rada and Leto, 2013). Elastases are hydrolytic enzymes known to affect the host cell proteins in infected tissues and facilitate bacterial invasion and growth (van der Plas et al., 2016). *T. natans* leaf extracts inhibited pyocyanin and elastase production at subinhibitory concentrations in a dose-dependent manner (Fig. 4). Anti-QS activity of *T. natans* leaves extracts has been similar as reported for *Mangifera indica L.* leaves extracts (Husain et al., 2017). At subinhibitory concentrations (100-400 μg/mL) methanol extract of *M. indica* leaves inhibited violacein production in *C. violaceum* up to 55%, as well as elastase and pyocyanin production in *P. aeruginosa* PAO1 up to 50% and 85%, respectively. Husain and coauthors also demonstrated inhibition of biofilm formation in *P. aeruginosa* PAO1 by 50%, but the effect was observed at the extract concentrations higher than 200 μg/mL. Similar anti-QS activity has been observed for various plants' extracts, but their active concentrations were usually higher than 1 mg/mL (Hossain et al., 2015; Yin et al., 2015).

Detailed chemical analysis of TnM performed in this study identified 22 different compounds. Thirteen of these compounds were identified in *T. natans* extracts for the first time. Phenolic acids such as ellagic, ferulic and gallic were found in higher amount in the

analyzed sample, while ellagic acid was identified as the most abundant component. The same phenolic acids were also determined in three different Chinese water caltrop cultivars, but gallic and ferulic acids were identified as the major components (Yu and Shen, 2015). Further, ferulic, caffeic, chlorogenic, and 3-O-methylgallic acids were previously detected in *T. natans* roots/leaves/pulp/hulls (Yu and Shen, 2015). Huang and coworkers identified lignans, tannins, and phenolic compounds such as naringenin, gallic acid, 4-hydroxybenzoic acid, and caffeic acid in ethyl acetate fraction from *T. natans* pericarps from Taiwan (Huang et al., 2016). Phenolic compounds such as gallic acid, caffeic acid, quercetin, and kaempferol were detected and quantified in green, red, and black fruit parts of *T. natans* var. *bispinosa* Roxb. from India (Niranjan et al., 2013).

The mechanistic study of QS inhibition by Tn extracts revealed that both TnM and TnA contain components with inhibitory effects on LasR and PqsR, and can stimulate RhlR *in P. aeruginosa* (Fig. 6). LasR regulates the production of elastases, alkaline protease, lipase, and LasI itself, while PQS system regulates pyocyanin production, biofilm formation, and PQS itself (Papenfort and Bassler, 2016). Both Las and PQS stimulate Rhl signaling pathway. RhlR system stimulates rhamnolipid production and biofilm formation, but represses PQS. The interconnection of QS signaling pathways in bacteria exposed to the mixtures of the compounds with QS agonistic or antagonistic activity provides fine tuning of the bacterial response to the stimuli and determines which of the virulence factors will be expressed and to which extent. Thus, inhibition of elastase and pyocyanin production in *P. aeruginosa* PAO1 cultured in the presence of TnM and TnA can be explained through their LasR and PqsR antagonistic activities, while stimulatory RhlR activity could be the cause of the increase of biofilm formation observed in the presence of TnM.

The inhibition of AHL-regulated gene expression and anti-QS activity of phenolic acids and their derivatives has been demonstrated earlier (Singh et al., 2009; Sarabhai et al., 2013; Borges et al., 2014; Myszka et al., 2016). Consistently, our results show that ellagic

and ferulic acids can inhibit Las signaling pathway through direct binding with specific receptor. In addition, it was shown that these phenolic acids could also bind Pqs receptor and interfere with PQS signaling (Fig. 7). The similar extent of the inhibition of Las and PQS pathways observed with the same concentrations of Tn extracts and the pure substances (Fig. 6 and 7) suggested their synergistic anti-QS activities. QS inhibitory activity of other, less represented compounds in *T. natans* leaf extracts should not be excluded and needs to be addressed in the future.

Herbal formulations and plant-derived therapeutics are generally less toxic than synthetic ones (Zimmer et al., 2012). However, the toxicity of high doses of plant drugs may be observed sometimes causing undesirable side effects. Hence, the cytotoxicity and toxicological risks of the Tn extracts were evaluated in this study using *in vitro* cell culture assay and *in vivo* zebrafish model. The zebrafish emerged as a versatile platform for drug discovery and toxicity assessment, due to its high genetic and molecular similarity to humans, and high correlation with humans in response to pharmaceuticals. This model system simplifies the path to clinical trials in humans and reduces the failure of potential therapeutics at later stages of testing (Chakraborty et al., 2009; MacRae and Peterson, 2015). The results showed that all three Tn extracts were cytotoxic to human lung fibroblast cell line in concentrations 40-50 μg/mL. However, TnM and TnA exhibited neither lethal nor developmental toxicity in zebrafish model up to 500 and 300 μg/mL, respectively, while TnEA extract showed *in vivo* toxicity when applied at bioactive concentrations (Fig. 5). Taken all together, the safe intake dose of TnM and TnA extracts that could exhibit anti-virulence effects would be 100 μg/mL.

In conclusion, the results of this study have shown that *T. natans* leaf extracts exhibit anti-QS activity in medically important pathogen *P. aeruginosa* reducing the production of various virulence factors, which are required for establishing infection. This is the first report showing that ellagic and ferulic acids could interfere with *P. aeruginosa* PQS signaling

pathway. The intake of food rich in phenolic acids and flavonoids has been associated with many biological effects and health benefit. The results of this study confirm the health benefit of *T. natans* extracts and explain its ethnopharmacological application against microbial infections.

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References

Abudoleh, S.M., and Mahasneh, A.M. (2017) Anti-Quorum Sensing Activity of Substances Isolated from Wild Berry Associated Bacteria. *Avicenna J Med Biotechnol* 9, 23-30.

Adkar, P., Dongare, A., Ambavade, S., and Bhaskar, V.H. (2014) *Trapa bispinosa* Roxb.: A Review on Nutritional and Pharmacological Aspects. *Adv Pharmacol Sci* 2014, 959830.

Allen, R.C., Popat, R., Diggle, S.P., and Brown, S.P. (2014) Targeting virulence: can we make evolution-proof drugs? *Nat Rev Microbiol* 12, 300-308.

Aloush, V., Navon-Venezia, S., Seigman-Igra, Y., Cabili, S., and Carmeli, Y. (2006) Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. *Antimicrob Agents Chemother* 50, 43-48.

Banjanac, T., Dragicevic, M., Siler, B., Gasic, U., Bohanec, B., Nestorovic Zivkovic, J., Trifunovic, S., and Misic, D. (2017) Chemodiversity of two closely related tetraploid Centaurium species and their hexaploid hybrid: Metabolomic search for high-resolution taxonomic classifiers. *Phytochemistry* 140, 27-44.

Bhardwaj, A.K., Vinothkumar, K., and Rajpara, N. (2013) Bacterial quorum sensing inhibitors: attractive alternatives for control of infectious pathogens showing multiple drug resistance. *Recent Pat Antiinfect Drug Discov* 8, 68-83.

Borges, A., Serra, S., Cristina Abreu, A., Saavedra, M.J., Salgado, A., and Simoes, M. (2014) Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and in vitro cytotoxicity. *Biofouling* 30, 183-195.

Bryers, J.D. (2008) Medical biofilms. *Biotechnol Bioeng* 100, 1-18.

Bystrom, L.M., Lewis, B.A., Brown, D.L., Rodriguez, E., and Obendorf, R.L. (2008) Characterization of phenolics by LC-UV/vis, LC-MS/MS and sugars by GC in *Melicoccus bijugatus* Jacq. 'Montgomery' fruits. *Food Chem* 111, 1017-1024.

Chakraborty, C., Hsu, C.H., Wen, Z.H., Lin, C.S., and Agoramoorthy, G. (2009) Zebrafish: a complete animal model for *in vivo* drug discovery and development. *Curr Drug Metab* 10, 116-124.

Chatterjee, M., Anju, C.P., Biswas, L., Anil Kumar, V., Gopi Mohan, C., and Biswas, R. (2016) Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. *Int J Med Microbiol* 306, 48-58.

Chiang, P.Y., Li, P.H., Huang, C.C., and Wang, C.R. (2009) Chemical composition and physical characteristics of water caltrop during growth. *Journal of the Science of Food and Agriculture* 89, 1298-1306.

Clinical and Laboratory Standards Institute (2012). M07-A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard: 9th ed. CLSI, W.P.

de Rijke, E., Out, P., Niessen, W.M., Ariese, F., Gooijer, C., and Brinkman, U.A. (2006) Analytical separation and detection methods for flavonoids. *J Chromatogr A* 1112, 31-63.

Djurdjevic, L. (2000) Content of phenolic acids and total phenolics in several aquatic plant species. *Arch Biol* 52 97-101.

Duan, K., and Surette, M.G. (2007) Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J Bacteriol* 189, 4827-4836.

Falcao, S.I., Vale, N., Gomes, P., Domingues, M.R., Freire, C., Cardoso, S.M., and Vilas-Boas, M. (2013) Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich in flavonoid glycosides. *Phytochem Anal* 24, 309-318.

Fletcher, M.P., Diggle, S.P., Crusz, S.A., Chhabra, S.R., Camara, M., and Williams, P. (2007) A dual biosensor for 2-alkyl-4-quinolone quorum-sensing signal molecules. *Environ Microbiol* 9, 2683-2693.

Frohlich, B., Niemetz, R., and Gross, G.G. (2002) Gallotannin biosynthesis: two new galloyltransferases from *Rhus typhina* leaves preferentially acylating hexa- and heptagalloylglucoses. *Planta* 216, 168-172.

Ha, D.G., Kuchma, S.L., and O'Toole, G.A. (2014) Plate-based assay for swarming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol* 1149, 67-72.

Hansen, M.B., Nielsen, S.E., and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119, 203-210.

Hossain, M.A., Lee, S.J., Park, J.Y., Reza, M.A., Kim, T.H., Lee, K.J., Suh, J.W., and Park, S.C. (2015) Modulation of quorum sensing-controlled virulence factors by *Nymphaea tetragona* (water lily) extract. *J Ethnopharmacol* 174, 482-491.

Huang, H.C., Chao, C.L., Liaw, C.C., Hwang, S.Y., Kuo, Y.H., Chang, T.C., Chao, C.H., Chen, C.J., and Kuo, Y.H. (2016) Hypoglycemic Constituents Isolated from *Trapa natans* L. Pericarps. *J Agric Food Chem* 64, 3794-3803.

Hummel, M., and Kiviat, E. (2004) Review of world literature on water chestnut with implications for management in North America. *Journal of Aquatic Plant Management* 42, 17-28.

Husain, F.M., Ahmad, I., Al-Thubiani, A.S., Abulreesh, H.H., AlHazza, I.M., and Aqil, F. (2017) Leaf extracts of *Mangifera indica* L. inhibit quorum sensing - regulated production of virulence factors and biofilm in test bacteria. *Front Microbiol* 8, 727.

Karg, S. (2006) The water chestnut (*Trapa natans* L.) as a food resource during the 4th to 1st millennia BC at Lake Federsee, Bad Buchau (southern Germany). *Environ Archaeol* 11, 125-130.

Kharbanda, C., Sarwar Alam, M., Hamid, H., Bano, S., Haider, S., Nazreen, S., Ali, Y., and Javed, K. (2014) *Trapa natans* L. root extract suppresses hyperglycemic and hepatotoxic effects in STZ-induced diabetic rat model. *J Ethnopharmacol* 151, 931-936.

Kim, B., Kim, J.E., Choi, B.K., and Kim, H.S. (2015) Anti-inflammatory effects of water chestnut extract on cytokine responses via nuclear factor-kappaB-signaling pathway. *Biomol Ther (Seoul)* 23, 90-97.

Koh, C.L., Sam, C.K., Yin, W.F., Tan, L.Y., Krishnan, T., Chong, Y.M., and Chan, K.G. (2013) Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors (Basel)* 13, 6217-6228.

Kordbacheh, H., Eftekhar, F., and Ebrahimi, S.N. (2017) Anti-quorum sensing activity of *Pistacia atlantica* against *Pseudomonas aeruginosa* PAO1 and identification of its bioactive compounds. *Microb Pathog* 110, 390-398.

Lavilla Lerma, L., Benomar, N., Casado Munoz Mdel, C., Galvez, A., and Abriouel, H. (2014) Antibiotic multiresistance analysis of mesophilic and psychrotrophic *Pseudomonas* spp. isolated from goat and lamb slaughterhouse surfaces throughout the meat production process. *Appl Environ Microbiol* 80, 6792-6806.

Lee, J., and Zhang, L. (2015) The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* 6, 26-41.

Lin, Q., Shen, J., Ning, Y., Shen, S., and Das, U.N. (2013) Inhibitory effects of water caltrop pericarps on the growth of human gastric cancer cells *in vitro*. *Curr Pharm Des* 19, 7473-7478.

MacRae, C.A., and Peterson, R.T. (2015) Zebrafish as tools for drug discovery. *Nat Rev Drug Discov* 14, 721-731.

Malviya, N., Jain, S., Jain, A., Jain, S., and Gurjar, R. (2010) Evaluation of in vitro antioxidant potential of aqueous extract of *Trapa natans* L. fruits. *Acta Pol Pharm* 67, 391-396.

Martinelli, D., Grossmann, G., Sequin, U., Brandl, H., and Bachofen, R. (2004) Effects of natural and chemically synthesized furanones on quorum sensing in *Chromobacterium violaceum*. *BMC Microbiol* 4, 25.

Massai, F., Imperi, F., Quattrucci, S., Zennaro, E., Visca, P., and Leoni, L. (2011) A multitask biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. *Biosens Bioelectron* 26, 3444-3449.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W.*, et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* 143 (Pt 12), 3703-3711.

Merritt, J.H., Kadouri, D.E., and O'Toole, G.A. (2005) Growing and analyzing static biofilms. *Curr Protoc Microbiol* Chapter 1, Unit 1B 1.

Mudric, S.Z., Gasic, U.M., Dramicanin, A.M., Ciric, I.Z., Milojkovic-Opsenica, D.M., Popovic-Dordevic, J.B., Momirovic, N.M., and Tesic, Z.L. (2017) The polyphenolics and carbohydrates as indicators of botanical and geographical origin of Serbian autochthonous clones of red spice paprika. *Food Chem* 217, 705-715.

Myszka, K., Schmidt, M.T., Bialas, W., Olkowicz, M., Leja, K., and Czaczyk, K. (2016) Role of gallic and p-coumaric acids in the AHL-dependent expression of flgA gene and in the process of biofilm formation in food-associated *Pseudomonas fluorescens* KM120. *J Sci Food Agric* 96, 4037-4047.

Niranjan, A., Verma, S., Lehri, A., and Amla, D.V. (2013) High-performance thin-layer chromatographic analysis for the simultaneous quantification of four phenolic compounds in green, red, and black fruits of *Trapa natans* var. bispinosa Roxb. (Singhara). *Jpc-Journal of Planar Chromatography-Modern Tlc* 26, 316-321.

Njoroge, J., and Sperandio, V. (2009) Jamming bacterial communication: new approaches for the treatment of infectious diseases. *EMBO Mol Med* 1, 201-210.

O'Loughlin, C.T., Miller, L.C., Siryaporn, A., Drescher, K., Semmelhack, M.F., and Bassler, B.L. (2013) A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc Natl Acad Sci U S A* 110, 17981-17986.

OECD guidelines for the testing of chemicals (2013). Fish embryo acute toxicity (FET) test. No. 236, O.P.

Ohman, D.E., Cryz, S.J., and Iglewski, B.H. (1980) Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. *J Bacteriol* 142, 836-842.

Overhage, J., Bains, M., Brazas, M.D., and Hancock, R.E. (2008) Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* 190, 2671-2679.

Paczkowski, J.E., Mukherjee, S., McCready, A.R., Cong, J.P., Aquino, C.J., Kim, H., Henke, B.R., Smith, C.D., and Bassler, B.L. (2017) Flavonoids suppress *Pseudomonas aeruginosa* virulence through allosteric inhibition of quorum-sensing receptors. *J Biol Chem* 292, 4064-4076.

Papenfort, K., and Bassler, B.L. (2016) Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol* 14, 576-588.

Pavlovic, A.V., Papetti, A., Dabic Zagorac, D., Gasic, U.M., Misic, D.M., Tesic, Z.L., and Natic, M.M. (2016) Phenolics composition of leaf extracts of raspberry and blackberry cultivars grown in Serbia. *Industrial Crops and Products* 87, 304-314.

Pellati, F., Orlandini, G., Pinetti, D., and Benvenuti, S. (2011) HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts. *J Pharm Biomed Anal* 55, 934-948.

Rada, B., and Leto, T.L. (2013) Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections. *Trends Microbiol* 21, 73-81.

Radojević, I.D., Vasić, S.M., Dekic, M.S., Radulović, N.S., Djelić, G.T., Djurdjević, J.S., and Čomić, L., R. (2016) Antimicrobial and antibiofilm effects of extracts from *Trapa natans* L., evaluation of total phenolic and flavonoid contents, and GC-MS analysis. *Acta Poloiae Pharmaceutica, Drug Research* 73, 1565-1574.

Razvy, M.A., Faruk, M.O., and Hoque, M.A. (2011) Environment friendly antibacterial activity of water chestnut fruits. *Jour Biodiv Environ Sci* 1, 26-34.

Ristivojevic, P., Trifkovic, J., Gasic, U., Andric, F., Nedic, N., Tesic, Z., and Milojkovic-Opsenica, D. (2015) Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC-LTQ/Orbitrap/MS/MS) study of phenolic profile of Serbian poplar type propolis. *Phytochem Anal* 26, 127-136.

Sanchez-Rabaneda, F., Jauregui, O., Casals, I., Andres-Lacueva, C., Izquierdo-Pulido, M., and Lamuela-Raventos, R.M. (2003) Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J Mass Spectrom* 38, 35-42.

Sarabhai, S., Sharma, P., and Capalash, N. (2013) Ellagic acid derivatives from *Terminalia chebula* Retz. downregulate the expression of quorum sensing genes to attenuate *Pseudomonas aeruginosa* PAO1 virulence. *PLoS One* 8, e53441.

Savic, N.D., Glisic, B.D., Wadepohl, H., Pavic, A., Senerovic, L., Nikodinovic-Runic, J., and Djuran, M.I. (2016) Silver(I) complexes with quinazoline and phthalazine: synthesis, structural characterization and evaluation of biological activities. *Medchemcomm* 7, 282-291.

Silva, L.N., Zimmer, K.R., Macedo, A.J., and Trentin, D.S. (2016) Plant natural products targeting bacterial virulence factors. *Chem Rev* 116, 9162-9236.

Singh, B.N., Singh, B.R., Singh, R.L., Prakash, D., Dhakarey, R., Upadhyay, G., and Singh, H.B. (2009) Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*. *Food Chem Toxicol* 47, 1109-1116.

Stoicescu, I., Popescu, A., Sirbu, R., and Bala, C. (2012) Simultaneous Determination of Phenolic Acids in Water Caltrop by Hplc-Dad. *Analytical Letters* 45, 2519-2529.

Vallverdu-Queralt, A., Jauregui, O., Medina-Remon, A., and Lamuela-Raventos, R.M. (2012) Evaluation of a method to characterize the phenolic profile of organic and conventional tomatoes. *J Agric Food Chem* 60, 3373-3380.

van der Plas, M.J., Bhongir, R.K., Kjellstrom, S., Siller, H., Kasetty, G., Morgelin, M., and Schmidtchen, A. (2016) *Pseudomonas aeruginosa* elastase cleaves a C-terminal peptide from human thrombin that inhibits host inflammatory responses. *Nat Commun* 7, 11567.

Vandana, B., Kavya, B., Shantha, T.R., Prathapa Reddy, M., Kavya, N., Rama Rao, V., Kalpeshkumar, B.I., and Venkateshwarlu, G. (2015) Pharmacognostical evaluation and phytochemical studies on Ayurvedic mutritional fruits *Trapa natans* L. *Int J Herb Med* 3, 13-19.

Wang, Y., Yang, J., Wang, A., Ma, J., Tian, J., Tengfei, J., and Su, Y. (2013) Hydrolyzable tannins from *Balanophora polyandra*. *Acta Pharmaceutica Sinica B* 3, 46-50.

Yin, H., Deng, Y., Wang, H., Liu, W., Zhuang, X., and Chu, W. (2015) Tea polyphenols as an antivirulence compound disrupt quorum-sensing regulated pathogenicity of *Pseudomonas aeruginosa*. *Sci Rep* 5, 16158.

Yu, H.N., and Shen, S.R. (2015) Phenolic composition, antioxidant, antimicrobial and antiproliferative activities of water caltrop pericarps extract. *Lwt-Food Science and Technology* 61, 238-243.

Zhu, F. (2016) Chemical composition, health effects, and uses of water caltrop. *Trends in Food Science & Technology* 49, 136-145.