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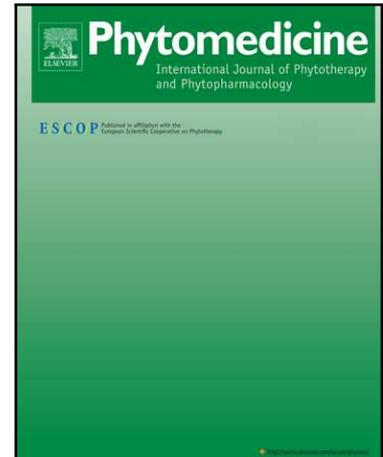
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## **Aqueous extract from *Orthosiphon stamineus***

### **leaves prevents bladder and kidney infection in mice**

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**ABSTRACT**

*Background:* Extracts from the leaves of *Orthosiphon stamineus* are used in phytotherapy for treatment of uncomplicated urinary tract infections.

*Purposes:* Evaluation of an aqueous extract against infection with uropathogenic *Escherichia coli in vivo*; investigation of underlying microbiological mechanisms.

*Study design:* *In vivo* studies in mice and *in vitro* investigations on cytotoxicity, antiadhesive potential, influence on bacterial gene expression and *quorum sensing*.

*Methods:* Extract OWE was prepared by hot water extraction. For *in vivo* studies BALB/c mice were used in an UPEC infection model. The effect of OWE on bacterial load in bladder/kidney tissue was monitored in pre- and posttreatment. Cytotoxicity of OWE against different UPEC strains, T24 bladder/A498 kidney cells, gene expression analysis, monitoring of phenotypic motility and *quorum sensing* was investigated by standard methods of microbiology.

*Results:* OWE was quantified (UHPLC) according to the content of rosmarinic acid, cichoric acid, caffeic acid. Three- and 5-day treatment of animals with OWE (750 mg/kg) after transurethral infection with UPEC CFT073 reduced the bacterial load in bladder and kidney, similar to norfloxacin. Four- and 7-day pretreatment of mice prior to the infection with UPEC NU14 reduced bacterial bladder colonization. *In vitro* investigations indicated that OWE ( $\leq 2$  mg/ml) has no cytotoxic or proliferation-inhibiting activity against different UPEC strains as well as against T24 bladder and A498 kidney cells. OWE exerts a dose dependent antiadhesive activity against UPEC strains NU14 and UTI89. OWE reduced gene expression of *fimH*, but evoked increase of the expression of motility/fitness gene *fliC*. Increase of bacterial motility on gene level was confirmed by a changed bacterial phenotype by an increased bacterial motility in soft agar assay. OWE inhibited in a concentration-dependent manner bacterial *quorum sensing*.

*Conclusion:* OWE is assessed as a strong antiadhesive plant extract for which the traditional use in phytotherapy for UTI might be justified.

*Keywords:* *Orthosiphon stamineus*, Adhesion, Antiadhesion, *Quorum sensing*, Uropathogenic *E. coli*, UPEC.

*Abbreviations:* AHL: acetylated homoserine lactone; BCR: Bacteria-cell ratio; FACS: fluorescence-activated cell sorting, flow cytometry; FimH: type 1 fimbriae, mannose sensitive; FliC: flagellum (H-antigen); IBC: intracellular bacterial communities; OWE: *Orthosiphon* aqueous extract;  $OWE^{\#tannin}$ : tannin-depleted *Orthosiphon* extract; PVPP: polyvinylpolypyrrolidone; 3OC<sub>6</sub>HSL: 3-oxohexanoyl-homoserine lactone; qRT-PCR: quantitative real time polymerase chain reaction; q.s.: *quorum sensing*; UHPLC: ultrahigh pressure liquid chromatography; UPEC: uropathogenic *E. coli*; UTI: uncomplicated urinary tract infections; VF: virulence factor.

## Introduction

Uncomplicated urinary tract infections (UTI) are one of the most common infectious diseases, caused in about 80 % of all cases by uropathogenic *Escherichia coli* (UPEC). The infectious process is mediated by a variety of bacterial virulence factors of UPEC, including adhesins, toxins, host defense evasion system, a specific iron acquisition system, and highly specialized proteins for improved adaptation to the host cell; Rafsanjany et al. (2015a) reviewed the most prominent virulence factors (VF), their specific functions and features.

During infection of the host cell initially specific cell surface-associated outer membrane proteins, the bacterial adhesins, enable UPEC to recognize complementary antigens of the eukaryotic cell and attach to the surface of the urinary epithelium. This step can result in bacterial invasion which subsequently leads to altered cell physiology and inflammatory response. Invasive UPEC may develop into biofilm-like so-called intracellular bacterial communities (IBCs) (for review see Dhakal et al. (2008). Additionally, UPEC-induced exfoliation of the host cells leads to a reduced barrier function and reduction of the bacterial load associated with the bladder epithelium (Hunstad and Justice, 2010).

Antibiotics are used as standard treatment for UTI, but antibacterial resistance and high recurrence rates emphasize the importance to develop alternative preventive strategies.

Extracts from the leaves of *Orthosiphon stamineus* BENTH. (syn. *O. aristatus* MIQ.) from the plant family Lamiaceae and native to tropical Asia, are recommended in traditional medicine for UTI. The use of *Orthosiphon* extracts for UTI within rationalized modern phytotherapy is also supported by official governmental monographs for formalized drug registration within the “Traditional Use” frame by the European Medicine Agency (HMPC Monograph, 2016). Also the European Scientific Cooperative on Phytotherapy highlights the use of *Orthosiphon* leave extracts (ESCOP monograph, 2009). The traditional use is summarized in the recommendations of HMPC, which mainly refers to water extract, but also hydroalcoholic extracts (ranking from 20 to 60% EtOH) are accepted for drug registration in EU.

The herbal material contains 0.5 to 0.7% flavonoids, especially methoxylated flavones (eupatorin, eupatorinmethylether, rhamnazin, scutellareintetramethylether, sinensetin, salvigenin) (Malterud et al., 1989), prenylated flavones (Yuliana et al., 2009), the flavonol eupatoretin, rosmarinic acid and di-caffeoyltartrat (Blaschek, 2015). Additionally the occurrence of diterpens, mainly the isopimaran derivatives orthosiphole A to Y (Nguyen et

al., 2004), seco-orthosiphole A to C, siphonol A to E 7, neio-orthosiphonon A 8, epi-orthosiphol N9, besides several triterpens and about 0.04% volatile oil are described (Bombardelli et al., 1972): Recent unpublished investigations indicated the presence of the methoxylated flavones, the depsides and the diterpens to be present in decoctions prepared from Orthosiphon leaves (Hensel and Sendker, 2016)

*In vitro* investigations on functional activity revealed a weak antibacterial and anti-inflammatory activity of Orthosiphon flavonoids by inhibition of lipoxygenase (Lyckander et al., 1992). Concerning potential effects on urine production published data reflect contradictory statements: Diuretic activity has been documented in animal experiments after application of Orthosiphon extracts to rabbits and dogs (Fevrier, 1933; Chow et al., 1979; Casadebaig et al., 1989) due to sinensetin (Chut and Zwaving, 1993) but clinical studies in humans did not prove significant diuretic activity (Nirdnoy and Muangman, 1991; Doan et al., 1992; Tiktinsky and Bablumyan, 1983).

From this point of view it might be speculated that the traditional use of Orthosiphon extract for UTI is not due to a pronounced diuretic effect, but more to an inhibitory effect against UPEC. During the last years new types of bacterial and viral inhibitors have been described, the so called antiadhesive drugs or also named as entry blockers. These compounds interfere with outer membrane proteins of Gram-negative bacteria, responsible for the specific recognition of surface antigens of the host cells and subsequent binding. This receptor-mediated adhesion process is the prerequisite for the subsequent internalization of the bacteria into the cell, intracellular proliferation, and the induction of host cell destruction and inflammatory response.

Antiadhesive compounds inhibit this highly specific docking process and lead to diminished infection of the tissue. Antiadhesive compounds have been described in the last years as new agents against infection with *Helicobacter pylori* (Messing et al., 2014), *Campylobacter jejuni* (Lengsfeld et al., 2007), Influenza A (Derksen et al., 2014), Herpes Virus I (Gescher et al., 2011) and also against UPEC (Rafsanjany et al., 2015a; Rafsanjany et al., 2015b). From this point of view, the present study aimed to investigate an aqueous extract from Orthosiphon leaves against UPEC in regard to potential direct cytotoxicity and antiadhesive potential within *in vitro* assays and *in vivo* animal experiments.

## Materials and methods

### *Solvents, reagents, general experimentation procedures*

If not stated otherwise, solvents, reagents, and consumables were obtained from VWR International (Darmstadt, Germany). All solvents and reagents were of analytical quality. Water was produced by a Millipore® Simplicity 185 system (Schwalbach, Germany). Dried leaf material from *O. stamineus* (*Orthosiphonis folium*), batch no 14017592, was obtained by Medice Arzneimittel Pütter GmbH & Co. KG (Isarlohn, Germany). The material was identified by A.H. and complied with the specification of European Pharmacopoeia (2014). A voucher specimen of the material is retained in the archives of Institute of Pharmaceutical Biology and Phytochemistry, Münster, Germany under the designation IPBP 406.

### *Preparation of Orthosiphon aqueous extract (OWE)*

Sixty grams of freshly powdered plant material were extracted 4 times with 800 ml of water for 2 h at 85 °C under continuous stirring for 8 h. The suspension was centrifuged for 15 min at  $5.000 \times g$ . The clear supernatant was concentrated by rotary evaporator, followed by lyophilization to yield 12 g of a slightly-brownish dry extract (herbal material : extract ratio = 5:1), which was termed as Orthosiphon water extract OWE. OWE was stored at -20 °C in sealed containers under vacuum.

### *UHPLC analysis of OWE*

Quantification of the extract and by using lead compounds of OWE was performed by ultrahigh pressure liquid chromatography (UHPLC).

Test solution: 50.0 mg of OWE were dissolved in 5.0 ml of water (Aqua Millipore) and filtered (Millex-LCR, Hydrophilic PTFE, 0.45  $\mu\text{m}$  VWR). This test solution was directly subjected to analytical UHPLC on Acquity™ Ultra Performance LC, PDA  $\lambda\text{e}$  Detector ( $\lambda = 210 - 400 \text{ nm}$ ), QDa™ mass-selective detector (negative and positive scan mass 100.00-1000.00 Da), autosampler, in-line degasser, Waters Empower 3®d (Waters, Milford, Milwaukee, U.S.A.) on an RP-18 stationary phase ACQUITY UPLC® HSS T3, 1.8  $\mu\text{m}$ , 2.1  $\times$  100 mm (Waters, Milford, Milwaukee, U.S.A.). Column temperature 40 °C. Mobile phase: binary gradient of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B):  $t_{0\text{min}}$  98% A,  $t_{14\text{min}}$  60% A,  $t_{14.1\text{min}}$  100% B,  $t_{15\text{min}}$  98% A. Flow rate: 0.5 ml/min. Injection volume: 2  $\mu\text{l}$ .

Reference compounds: rosmarinic acid ( $t_R = 8.8$  min) (Roth, Karlsruhe, Germany), (purity HPLC 91% at  $\lambda = 215$  nm); caffeic acid ( $t_R = 5.1$  min) (Roth, Karlsruhe, Germany) (purity HPLC 100% at  $\lambda = 215$  nm); cichoric acid ( $t_R = 7.5$  min), isolated from *Echinodorus grandiflorus* (Schnitzler, 2005) (purity HPLC = 95% at  $\lambda = 215$  nm)

*Preparation and UHPLC-characterization of tannin-depleted Orthosiphon aqueous extract (OWE<sup>#tannin</sup>)*

Two hundred grams of OWE were dissolved in 500 ml water. Ten grams of Polyclar AT (G. A. F. Corporation, USA), a water-insoluble polyvinylpyrrolidone, were added. Polyphenols were precipitated under gentle stirring of the reaction vessel for 60 min. The suspension was centrifuged at  $3000 \times g$  for 5 min. The supernatant was filtered through a paper filter and clear filtrates were combined, evaporated, and lyophilized to yield 20 mg of OWE<sup>#tannin</sup>, which was subsequently characterized by the UHPLC method described above.

*Uropathogenic E. coli (UPEC) strains and growth conditions*

Bacterial strains: UPEC strains NU14 and UTI89 (both clinical isolates obtained from cystitis patient and a patient with an acute bladder infection respectively) (Johnson and Weissman, 2001; Chen et al., 2006), UPEC pyelonephritis isolate CFT073(O6:K2:H1) (Guyer et al., 1998), *E. coli* 2980 (DSM 10791) (Hacker and Ott, 1986) were used in this study. Bacteria from the second passage were used for all tests and cultivated from frozen stocks.

Agar grown bacteria were harvested and used for inoculation of Loeb agar, supplemented with 0.2% CaCl<sub>2</sub> which is supposed to increase the type 1 fimbria expression (Connel et al., 1996). For liquid culture one colony of agar grown bacteria was transferred to 10 ml of LB medium or pooled human urine supplemented with 5% of LB medium. The cultures were incubated in 50 ml Falcon tubes under shaking (200 rpm/37 °C) for 17 h.

*Cell culture, agar diffusion assay, monitoring of bacterial growth in liquid culture, determination of cell viability (MTT assay), and adhesion assay by quantitative flow cytometry* was performed as described by Rafsanjany et al. (2015a).

*Isolation, quantitation and reverse transcription of total RNA*

T24 cells were seeded in six well plates at a density of  $2.5 \times 10^5$  cells per well. UPEC strain NU14 (1 CFU from agar grown bacteria) were cultivated for overnight in pooled human urine at 37 °C under shaking (180 rpm), supplemented with 5% LB medium and OWE (2 mg/ml); the respective control group did not contain the plant extract. Bacteria were pelleted ( $6.000 \times g$ ) and resuspended in 1 ml DMEM medium. The OD was adjusted to 4 (dilution factor integrated into the calculation). 100  $\mu$ l of this bacterial suspension are mixed with 900  $\mu$ l of DMEM. 1000  $\mu$ l of the resulting suspension was added to the T24 host cells in 6 well plates. The mixture was incubated for 60 min at 5% CO<sub>2</sub>/37 °C. The supernatant, containing unattached bacteria, was removed by use of a pipette and stored until further use in 1.5 ml plastic tubes. The T24 cell monolayer was scraped off by use of a Cell Scraper (Sarstedt, Germany), suspended in 1 ml of phosphate buffered saline (PBS) and transferred to 1.5 ml plastic tubes. The suspension was centrifuged ( $10.000 \times g$ , 5 min). The supernatant was removed and the pellet resuspended in 1.0 ml PBS.

Total cellular RNA was isolated from both samples, the unattached bacteria in the incubation supernatant and from the T24 host cells with attached UPEC. For RNA isolation RNeasy Mini Kit (Qiagen, Germany) was used according to the manufacturer's instructions. RNA quality and concentration was determined using the  $\mu$ Cuvette G1.0 and the BioPhotometer plus (both Eppendorf, Germany). Transcription into cDNA was achieved by the use of the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to manufacturer's instructions.

*Quantitative Real Time PCR (qRT-PCR)*

qRT-PCR was performed with an equivalent of 15 ng of total RNA by using the iTaq Universal SYBR Green supermix (BioRad, Germany) according to the protocol recommended by the manufacturer and the CFX96 Real-Time SystemC1000 Touch (BioRad, Germany). The qPCR parameters were as follows: polymerase activation and initial denaturation for 15 s at 95 °C followed by 39 cycles of 5 s at 95 °C and 30 s at 60 °C. Afterward, an additional melt curve analysis was performed (65–95 °C, ramp 0.5 °C pro cycle, 1 cycle = 5 s, 60 cycles in total). Data were evaluated with the BioRad CFX Manager 3.0 software based on the comparative CT method (2 $^{-\Delta\Delta CT}$  method) and normalized to the endogenous reference gene

coding for the 16S rRNA. Primers for the qPCR were designed with the Universal Probe Library Assay design Center (Roche, Switzerland), and the oligonucleotides were obtained from Eurofins MWG Operon, Luxembourg. Primer sequences used for the differential gene expression analysis are listed in Table 1.

#### *Motility assay*

Motility was evaluated using soft-agar plates (1% tryptone, 0.5% NaCl, 0.25% and 0.5% agar), which were prepared the day prior to use and left at room temperature overnight. UPEC strain NU14 (one colony from agar grown bacteria) were cultivated overnight in pooled human urine at 37 °C under shaking (200 rpm), supplemented with 5% liquid medium and OWE (2 mg/ml); the respective control group did not contain the plant extract. The cultures were stabbed into the middle of the soft-agar plates using a sterile inoculating needle (it was paid attention not to touch the bottom of the plate so as to avoid possible twitching motility). Plates were incubated at 30 °C for approximately 17 h depending on the amount of motility. Motility was determined by viewing wet mounts of bacterial cultures and measuring the radius of motility.

#### *Quorum sensing inhibition activity*

To examine the *quorum sensing* inhibition activity of OWE a transformed *E. coli* biosensor reporter strain was used as reported in a recent study (Vila-Sanjurjo (submitted)). *E. coli* strain Top10 was transformed by use of plasmid pSB1A3 - BBa\_T9002, carrying the BBa\_T9002 genetic device (Registry of Standard Biological Parts: [http://parts.igem.org/Part:BBa\\_T9002](http://parts.igem.org/Part:BBa_T9002)) ligated into vector pSB1A3, introduced by chemical transformation and stored as a 30% glycerol stock at -80°C. The sequence BBa\_T9002, comprised the transcription factor (*LuxR*), which is constitutively expressed but it is active only in the presence of the exogenous cell-cell signaling molecule 3-oxohexanoyl-homoserine lactone (3OC<sub>6</sub>HSL). At an adequate concentration, two molecules of 3OC<sub>6</sub>HSL bind to two molecules of LuxR and activate the expression of GFP (output), under the control of the *lux* pR promoter from *Vibrio fischeri*.

*Growth media and conditions:* Bacterial strains were cultivated on LB7 and M9 minimal medium (Becton, Dickinson, Heidelberg, Germany). 10 ml of LB broth, supplemented with 200 µg/ml ampicillin were inoculated with one colony from a freshly streaked plate of Top10 containing BBa\_T9002 and incubated for 18 h at 37°C under shaking at 100 rpm. Each culture was then diluted 1:1000 into 20 ml M9 minimal medium supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride plus 200 µg/ml ampicillin (AppliChem,

Germany). The culture was maintained under the same conditions until the  $OD_{600nm}$  reached 0.15 (~ 5 h). Then, 500  $\mu$ l of overnight culture were mixed with 500  $\mu$ l of 30% sterile glycerol and stored at  $-80$  °C. Prior to the biosensor assay, 40  $\mu$ l of the overnight culture were cultivated in 20 ml M9 medium in the presence of 200  $\mu$ g/ml ampicillin. Once the culture  $OD_{600nm}$  reached OD 0.04 to 0.07 (~ 4 h), it was considered as ready for the biosensor assay.

*E. coli* Top10 biosensor assay. The 3OC<sub>6</sub>HSL was dissolved in acetonitrile to a stock concentration of 100 mM and stored at  $-20$  °C until usage. Prior to each experiment serial dilutions from the stock solution were prepared in water to produce solutions with a concentration ranging from 10 nM to 100 mM. 10  $\mu$ l of 3OC<sub>6</sub>HSL solution were mixed with 10  $\mu$ l of OWE (1, 5, 10 and 20 mg/ml) in the wells of a flat-bottomed 96-well plate (Greiner Bio-One, Germany), and each well was then filled with 180  $\mu$ l aliquots of the bacterial culture to test for QS inhibition activity. Several controls were also included. Blank 1 contained 180  $\mu$ l of M9 medium and 20  $\mu$ l of MilliQ water to measure the absorbance background. Blank 2 contained 180  $\mu$ l of bacterial culture and 20  $\mu$ l of MilliQ water, thus allowing measuring the absorbance of background corrected for the cells. Finally, the positive control wells contained 10  $\mu$ l of water plus 10  $\mu$ l 3OC<sub>6</sub>HSL solution and 180  $\mu$ l of the bacterial culture to measure of the fluorescence background. In order to allow for the effect of OWE themselves on  $OD_{600}$  and fluorescence, 10  $\mu$ l of 3OC<sub>6</sub>HSL solution were mixed with 10  $\mu$ l of OWE in the wells and each well was then filled with 180  $\mu$ l M9 medium, and it was measured in identical conditions as for the rest of the assays.

The plates were incubated in a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany) at 37 °C and fluorescence measurements were registered automatically using a repeating procedure ( $\lambda_{ex/em}$  = 480/510 nm, 40  $\mu$ s, 10 flashes, gain 100, top fluorescence), absorbance measurements ( $OD_{600nm}$ ) ( $\lambda$  = 600 nm absorbance filter, 10 flashes) and shaking (5 s, orbital shaking, high speed). The interval between the measurements was 6 min. For each experiment, the fluorescence intensity (FI) and  $OD_{600nm}$  data were corrected by subtracting the values of absorbance and fluorescence backgrounds and expressed as the average for each treatment. All measurements consisted in a minimum of three biological replicates.

#### *In vivo infection experiments: Mice infection model*

The study was performed at Pasteur Institute of Iran according the international guidelines for animal studies and instructions of the local ethical committee (allowance number

IR.PII.REC.1394.80). Female BALB/c mice, 20 to 22 g and aged 6-8 weeks were obtained from the breeding station of Pasteur Institute, Karaj, Iran, and were maintained on a 12 h light/dark cycle, at constant temperature at 21 °C and with free access to water and standard dietary food. Treatment of the animals: application of test/control solutions was performed by intragastric application of 500 µl of the respective test solutions (untreated control: water; treated groups: OWE at different concentrations, positive control: norfloxacin 100 mg/kg body weight/day). Gavage was performed using a 20 gauge-feeding needle 1465LL (Acufirm Ernst Kratz, Dreieich, Germany). The test extract OWE was dissolved in distilled water for preparation of the test solution. Animals were randomly distributed into different treatment groups. Testing of OWE was performed by pre- or posttreatment of the animals prior or after the infection with two different *E. coli* strains over different time intervals with determination of the bacterial load in bladder and/or kidney. A detailed overview of the different treatment protocols and animal groups is displayed in Table. 2.

For pretreatment mice were infected 6 h after the last dose of OWE with  $2 \times 10^8$  NU14 or  $1 \times 10^8$  CFT073. For posttreatment mice were infected with UPEC CFT073 ( $1 \times 10^8$  CFT073) and after 12 h the test compounds/extract was administered at different concentrations (Tab. 2). The positive control group was treated with norfloxacin (100 mg/kg body weight/day) in the posttreatment protocol for either 3 or 5 consecutive days after infection at day 0 (please note: pretreatment of animals with norfloxacin in parallel to the OWE pretreatment protocol did not prevent the infection, and therefore no positive control group could be performed for the pretreatment cycle).

The infection of the animal was performed according to the method described by Lane et al., (2005). Animals were anesthetized with ketamine/xylazine (70 mg/kg + 5 mg/kg) (Alfasan, Netherland) and inoculated transurethrally with 20 µl of the respective UPEC suspension, containing either  $2 \times 10^8$  CFU NU14 or  $1 \times 10^8$  CFU CFT073 per mouse by using a sterile polyethylene catheter PE-10 (inner diameter 0.28 mm; outer diameter 0.61 mm) (Warner Instruments, U.S.A.) After the experiments mice were sacrificed by means of cervical dislocation. The bladder and kidneys were aseptically removed and homogenized in 1.0 ml of PBS in a sterile 1.5 ml Micro Tube by using either a G50 Tissue Grinder (Coyote Bioscience Inc. Columbia, MD, U.S.A.) at a grinding speed of 8 000 rpm for 10 sec for 3 consecutive times or 2 ml Dounce Tissue Grinder™ (B. Braun Biotech, International).

The resulting suspension was spiral-plated onto plain LB agar plates, followed by incubation for 24 h at 37 °C. CFU per ml homogenate and per animal tissue were counted and quantified for each tissue of each animal.

A group size of  $n = 6$  mice was used for untreated controls (UC) and OWE treated groups while for positive control (PC) only 3 animals were used.

#### *Statistical analysis*

One-way ANOVA, Student's t-test, and the Tukey HSD test were used to compare the differences between the mean values of the groups. The results of infection experiments were also analyzed by Kruskal–Wallis test with Dunn's multiple comparisons, using GraphPad Prism software.  $p < 0.05$  of all results was considered as significant.

### **Results and Discussion**

Extract OWE from *O. stamineus* was obtained in a yield of 20% (w/w, related to the starting material; herbal material/extract ratio = 1:5). For quantification a UHPLC method was established and validated according to the ICH2 guidelines (ICH-Q2 (R1) guidelines, 1996) concerning the relevant parameters specificity, linearity, repeatability and accuracy for the three lead compounds caffeic acid, cichoric acid and rosmarinic acid. A representative UHPLC chromatogram of OWE is displayed in Fig. 1. The content of the three lead compounds in OWE was determined as  $7.1 \pm 0.3$  mg/g caffeic acid,  $7.9 \pm 0.4$  mg/g cichoric acid and  $10.9 \pm 0.4$  mg/g rosmarinic acid.

Direct cytotoxicity of OWE against UPEC strain NU14 and *E. coli* 2980 was monitored in the concentration range between 0.1 to 2 mg/ml in agar diffusion assay over 24 h (data not shown) and in liquid culture over 6 h (see Supplementary Data, Fig. S1A and S1B). No inhibition of bacterial proliferation was observed in both systems. From this it can be concluded that OWE has no toxic influence on UPEC within the tested concentrations.

Influence of OWE against eukaryotic host cells (T24 bladder and A498 kidney cells) was investigated by monitoring cell viability via mitochondrial activity (Mosmann, 1983). A slightly reduced cell viability to  $\geq 80\%$ , related to the untreated control groups, was observed in both cell lines at 1 and 2 mg concentration (see Supplementary Data Fig. S2). This effect is assessed as not relevant to claim a strongly disturbed cell physiology or even toxic effects, but

is seen as a more or less unspecific effect at these quite high concentrations. From that it can be concluded that OWE has no relevant toxic influence on epithelial cells. All further studies on antiadhesive effects of OWE were performed at reduced contact times (90 min) of OWE with the eukaryotic cells.

Subsequently, the influence of OWE on UPEC adhesion on eukaryotic host cells was investigated. The detailed influence of the test compounds can be assessed by using different incubation protocols, with coincubation (UPEC + host cell + test extract, all together) or preincubation of UPEC (UPEC + test extract in human pooled urine, followed by incubation of the pretreated bacteria together with host cells).

During the initial experiments it turned out that in case of coincubation OWE increased the bacterial attachment up to 300%, related to the untreated control (see Supplementary File Fig. S3).

Microscopic investigation of the so obtained cell cultures after Giemsa staining indicated the presence of OWE-induced UPEC clusters, strongly attached to the T24 cells (see Supplementary File Fig. S4A and S4B).

A similar observation during such *in vitro* adhesion assays with polyphenol-enriched plant extracts has recently been published also for Cranberry extracts (Rafsanjany et al., 2015a), indicating that such experimental setups do not lead to meaningful results. As OWE contains a high amount of polyphenols from which especially the rosmarinic acid and the cichoric acid as typical depsides are known to have tannin-like astringent properties it was concluded that these compounds should be responsible for the clustering and agglomeration of the bacteria to the host cells. For that it was decided that *in vitro* adhesion experiments in the coincubation mode with tannin-containing OWE are meaningless.

Therefore polyphenols were removed from OWE by treatment of the extract with insoluble polyvinylpolypyrrolidon (PVPP), a polymer known to bind polyphenolic compounds (Rafsanjany et al, 2015a) to obtain a polyphenol-depleted extract OWE<sup>∓tannin</sup>. Strong reduction of the phenolic compounds was proven by UHPLC analysis, indicating only residual contents of rosmarinic acid, cichoric acid and caffeic acid; the content of the three lead compounds in OWE<sup>∓Tannins</sup> was determined with  $0.1 \pm 0.02$  mg/g caffeic acid,  $0.2 \pm 0.02$  mg/g cichoric acid and  $0.2 \pm 0.02$  mg/g rosmarinic acid.

Using this tannin-depleted extract OWE<sup>z/tannin</sup> in the coincubation adhesion assay no agglomeration and cluster formation between host cells and UPEC was observed anymore. At concentrations  $\geq 0.5$  mg/ml a slight, but not significant reduction in bacterial adhesion was observed (Data not shown). At this stage of the investigations it is not clear what compounds form OWE are responsible for the observed antiadhesive effect, and further studies have to be performed to identify the active compounds by bioassay guided.

Different results were obtained within preincubation of UPEC with both tested extracts (Fig. 2). In case of preincubation of the bacteria with OWE (1 and 2 mg/ml) the adhesion of UPEC strain NU14 to T24 cells was significantly reduced by about 40%. The tannin-depleted extract OWE<sup>z/tannin</sup> evoked a concentration-dependent inhibition of bacterial adhesion (Fig. 2). From these data it can be concluded that aqueous Orthosiphon leaf extract seems to have an antiadhesive potential against UPEC under *in vitro* conditions. Similar well documented antiadhesive effects have been reported only for a few selected medicinal plants; the most prominent species, also used as food supplement for prevention of UTI, is *Vaccinium macrocarpon*, from which the mature fruits (Cranberries) are used and for which *ex vivo* antiadhesive effects of the urine after consumption in humans has been described recently (Rafsanjany et al., 2015a). Also strong *in vitro* antiadhesion against UPEC has been documented for extracts from *Zea mays* with derhmanosylmaysin derivatives probably to be the active ingredients (Rafsanjany et al., 2015b). Some other plant species have been reported to inhibit the bacterial adhesion to human bladder cells within a survey of traditionally used plants, with *Solidago* sp., and *Ononidis* sp., which are often parts of decoctions widely used in the European population for UTI (Rafsanjany et al., 2013).

To correlate these *in vitro* data with a potential *in vivo* activity an UPEC infection model in mice was used. In principle, animals were infected transurethrally with either UPEC strain NU14, known to interact with bladder tissue, or strain CFT073, known to be associated with the clinical symptoms of nephritis (Rudick et al., 2010; Mobley et al., 1990).

Within a first experimental series two different animal groups were pretreated for 4 days intragastrically with OWE at concentrations of 500 resp. 750 mg/kg body weight/day (Table 2); additionally, one group was pretreated for 7 days with 750 mg/kg/day of OWE, followed by infection with UPEC strain NU14 ( $2 \times 10^8$  bacteria). 24 h post infection animals were sacrificed, the bladder tissue was removed, homogenized and the bacterial load in the tissue was quantified. As displayed in Fig. 3 the 4-day pretreatment evoked a strong reduction in bacterial load in bladder tissue from about  $10^6$  to about  $10^4$  colonies per bladder. No

significant difference was observed between the 500 and 750 mg/kg treated OWE groups during the pretreatment for 4 days.

Significant reduction in bacterial load was observed for the group pretreated for 7 days, for which a reduction to about below  $10^3$  colonies was determined (Fig. 3).

It was not possible to use a standard antibiotic treatment (positive control) in this protocol as a 4 day pretreatment of the animals with norfloxacin did not have any influence on the bacterial load and therefore could not be used (data not shown). Instead the suitability of the test system was validated by the use of a 4 day posttreatment with norfloxacin which significantly reduced bacterial load (data not shown).

From these data a positive effect of the pretreatment of mice with OWE prior to infection with strain NU14 was obvious.

Within a second experimental series OWE was tested at 750 mg/kg/day intragastrically against CFT073 infected animals. Both, the bladders as well as the kidneys were analyzed separately for the respective bacterial load (Table 2). As shown in Fig. 4A 7 day pretreatment with OWE led to a reduction of the mean UPEC colonization in the bladder tissue, which did not turn out to be significant due to the high variability (note for Fig. 4A: for the treated group “pre-OWE – bladder” only 5 mice were included into the final evaluation, as one animal died for bladder injury during the bacterial inoculation). Similar results, but also not significant, were obtained during quantification of the bacterial load in the kidneys (Fig. 4A) (note for Fig. 4A: for control group UC only five animals could be finally evaluated due to contamination of an agar plate).

A 3 day post infection treatment with OWE reduced the bacterial colonization in bladder tissue, similar to those determined for the positive control norfloxacin (Fig. 4B). The same was true for the bacterial load in the kidneys (Fig. 4B) (note for Fig. 4B: for the treated groups “OWE” only 5 mice were included into the final evaluation, as one animal died; as the reason for this remained unclear the respective data have not been integrated into the evaluation).

Posttreatment over a 5 day period (Fig. 4C) did not change the bacterial load in the bladder tissue to a higher extent compared to the 3 day treatment with OWE (Fig. 4B), but in case of quantitation of UPEC infection in the kidneys the 5 day posttreatment resulted in the complete absence of bacterial in the kidney (Fig. 4C).

From these data it can be concluded that the posttreatment of the animals with OWE after infection can reduce infection severity of UPEC in mice significantly and especially the infection of kidneys after a bladder infection might be effectively prevented. This finding is assessed to be in good congruence with the observed antiadhesive effects from the *in vitro* experiments and indicates that the ascending infection of UPEC along the urogenital epithelia is strongly reduced by OWE, probably due to the antiadhesive effects of the extract.

At this stage the question on the underlying molecular mechanisms for the antiadhesive effect arises. On the one hand it might be that compounds from OWE directly interact with the bacterial adhesins, but also an altered phenotype of the bacteria by OWE is possible, which again can be monitored by gene and protein expression analysis. Therefore, gene expression of two selected virulence factors from UPEC strain NU14 was monitored by quantitative PCR: the FimH adhesin of strain NU14, which represents the mannose sensitive, uropilin-binding protein, mainly responsible for recognition and attachment to and invasion into the host cell, and the FliC flagellin protein, required for bacterial motility. For the gene expression assay UPEC NU14 was pretreated with OWE (2 mg/ml) in pooled human urine. After removal of the extract the bacteria were incubated together with T24 bladder cells. T24 cells with attached bacteria were separated from free-floating bacteria in the supernatant. Gene expression was separately monitored for bacteria attached to the T24 host cells and for non-attached, free floating bacteria in the supernatant.

OWE pretreatment evoked about 50% reduced *fimH* transcript compared to the untreated control in case of bacteria which were attached to T24 cells (Fig. 5), while the expression of *fliC* was slightly upregulated. In case of the free floating, non-attached bacteria no change of *fimH* mRNA expression was observed in comparison to the untreated cells, but OWA induced an about 6-folds upregulation of *fliC* mRNA in the free floating bacteria (Fig. 5). From these data it can be deduced that compounds from OWE downregulate *fimH* expression, leading to a reduced attachment to the host cells. As a result of this “attachment deficiency” the motility gene *fliC* is upregulated in order to achieve improved contact with potential surface structures for adherence.

For investigation if this influence of OWE on gene expression level is also reflected by changes in the bacterial phenotype a motility assay was performed, which is strongly related to the activity of the flagellin FliC (Haiko and Westerlund-Wikström, 2013). Therefore bacteria, cultivated in pooled human urine with or without OWE (2 mg/ml) were stabbed on semi-solid agar (0.25% and 0.5%). As it is known that bacteria will disperse in semi-solid

agar solutions not only on top (as they do on solid agar 1%), but also insert and move inside and through the soft agar, causing the matrix to appear turbid, an increased fitness and motility of the bacteria will result in different spreading behavior in the plate (Lane et al., 2005). As displayed in Fig. 6 OWE-pretreated UPEC showed higher motility compared to the untreated bacteria. Thus, the gene expression analysis, which indicates increased *fliC* expression can be correlated with a changed phenotypic behavior.

As OWE has an influence on the UPEC geno- and phenotype it was worth to investigate also the influence of the extract on bacterial cell-cell communication by *quorum sensing*, which is mediated by small molecular signals that are termed autoinducers (Waters and Bassler, 2005). *Quorum sensing* regulates the gene expression in response to the changes of cell-population density and controls many physiological activities, including virulence factor production, motility and biofilm formation (Miller and Bassler, 2001). To investigate a potential influence of OWE on *quorum sensing* an *E. coli* Top 10 reporter biosensor strain was used. This strain constitutively expresses the LuxR receptor which is only active in the presence of the exogenous cell-cell signaling molecule 3OC<sub>6</sub>HSL. At an adequate concentration, two molecules of 3OC<sub>6</sub>HSL will bind to two molecules of LuxR and activate the expression of GFP (output), under the control of the *lux* pR promoter from *Vibrio fischeri*. As shown in Fig. 7 a concentration-dependent inhibition of *quorum sensing* was observed for OWE-treated *E. coli*, which was evidenced as the reduction on bacterial fluorescence intensity by  $28.5 \pm 7.8\%$  at 0.5 mg/ml and by  $47.9 \pm 10\%$  at 1 mg/ml. At the same time, OWE had no effect on bacterial proliferation. Therefore these results demonstrate that OWE has a strong inhibition activity of *quorum sensing* activity regulated by AHL.

## Conclusion

Results presented in this study demonstrate that the traditional use of aqueous extracts from *O. stamineus* is due to a concentration-dependent antiadhesive activity against UPEC. Orthosiphon extract is active after oral intake in mice, which implies that the active constituents of the extract are absorbed from the intestinum into the systemic compartment. Besides blocking of the bacterial adhesion factors the extract also has a strong effect on bacterial gene expression and phenotypic behavior. From our point of view the clinical use of aqueous Orthosiphon extracts might be rationalized, but more detailed clinical studies in humans should be performed.

### **Acknowledgments**

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### **Conflict of interest**

The authors have declared no competing conflict of interest.

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### **Author's contribution**

SB performed the experiments, SB provided assistance to gene expression experiments, XFQ and FMG performed the *quorum sensing* studies, UD provided UPEC strains and advised microbiological investigations, AK, HM and BS monitored the *in vivo* studies, AH designed and mentored the study and wrote the manuscript.

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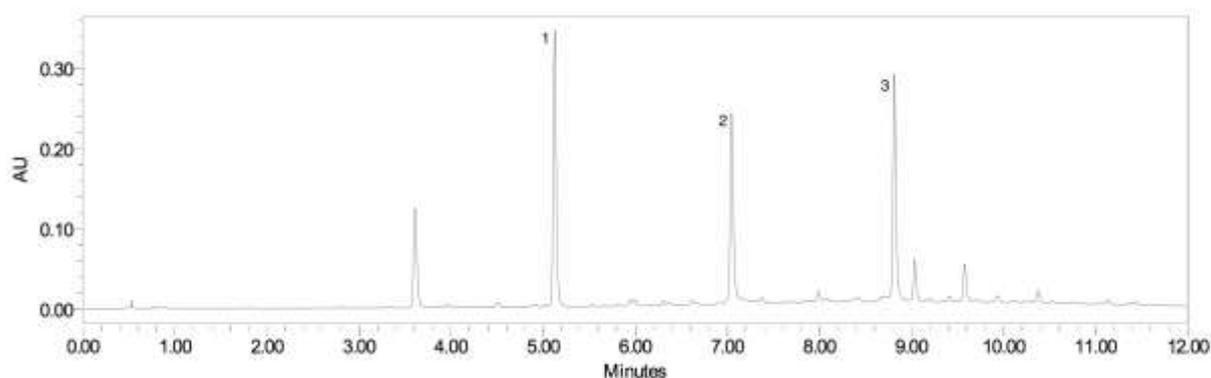
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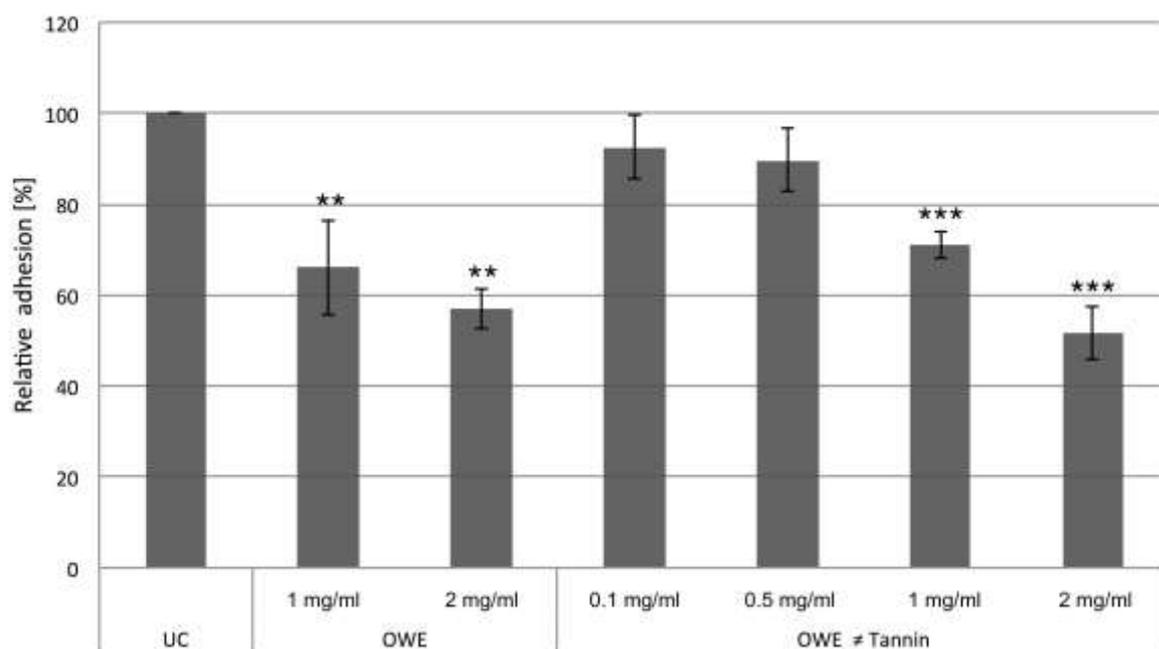
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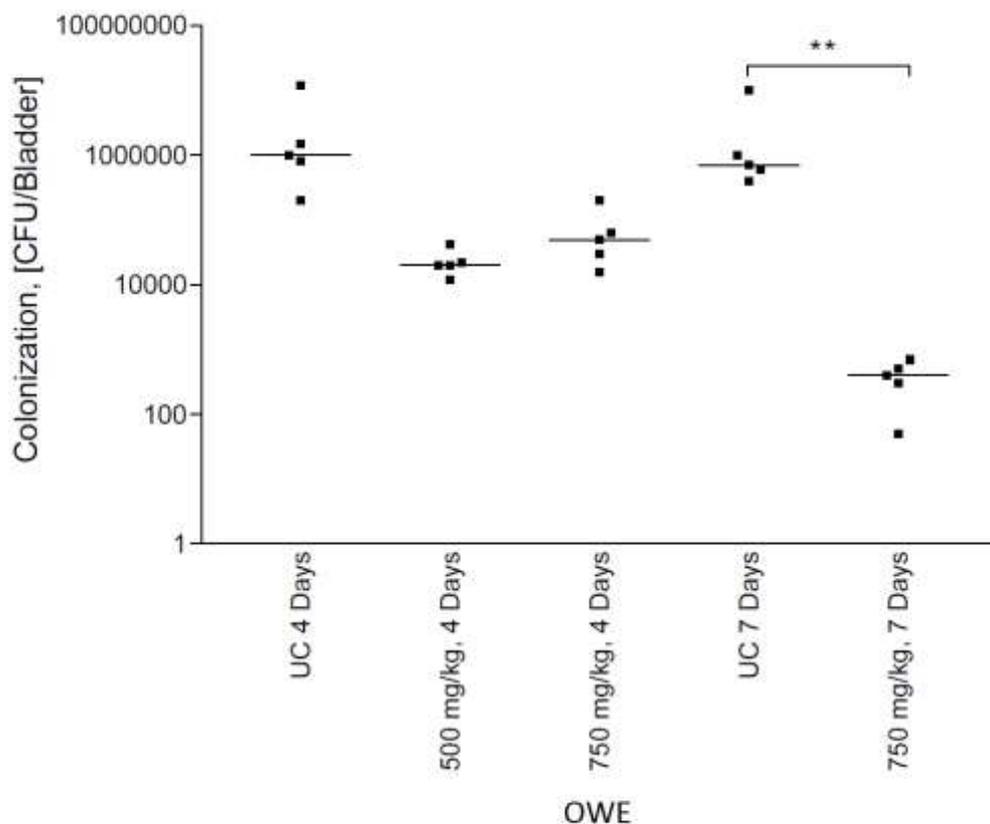
## Figure legends



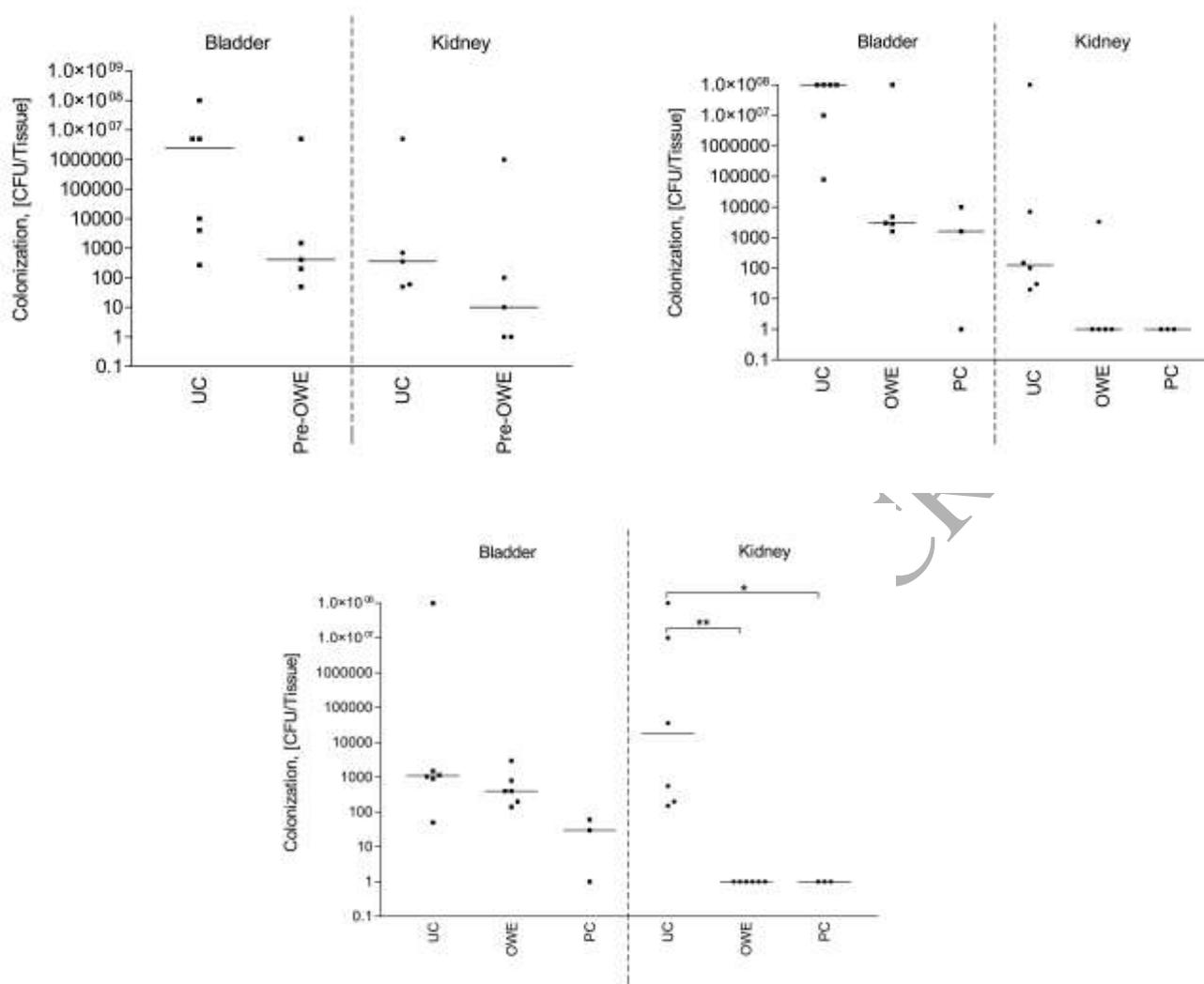
**Fig. 1:** Representative UPLC chromatogram of OWE with caffeic acid (1), cichoric acid (2) and rosmarinic acid (3); UV detection  $\lambda = 330$  nm.



**Fig. 2:** Influence of different concentrations of OWE and OWE<sup>without tannin</sup> on the relative adhesion of FITC-labeled *E. coli*, strain NU14 to T24 bladder cells after 90 min of preincubation of the bacteria with the test extracts. Data indicate the adhesion related to the untreated control (UC = 100%). \*\*  $p < 0.01$ . \*\*\*  $p < 0.001$ . Values represent the mean  $\pm$  SD of three independent experiments.



**Fig. 3:** Influence of a 4 and 7 days pretreatment of female Balb/c mice with OWE (500 and 750 mg/kg body weight) on the bacterial load in bladder after transurethral infection of the animals at day 0 with  $2 \times 10^8$  cells of UPEC strain NU 14; animals were sacrificed 24 h after infection, complete bladder had been removed, homogenized and the bacterial load was determined by quantification of the colony forming units (CFU) after plating on LB agar. Each spot represents the CFU/bladder from one animal; bars represent the mean value. \*  $p < 0.1$ . \*\*  $p < 0.01$ .



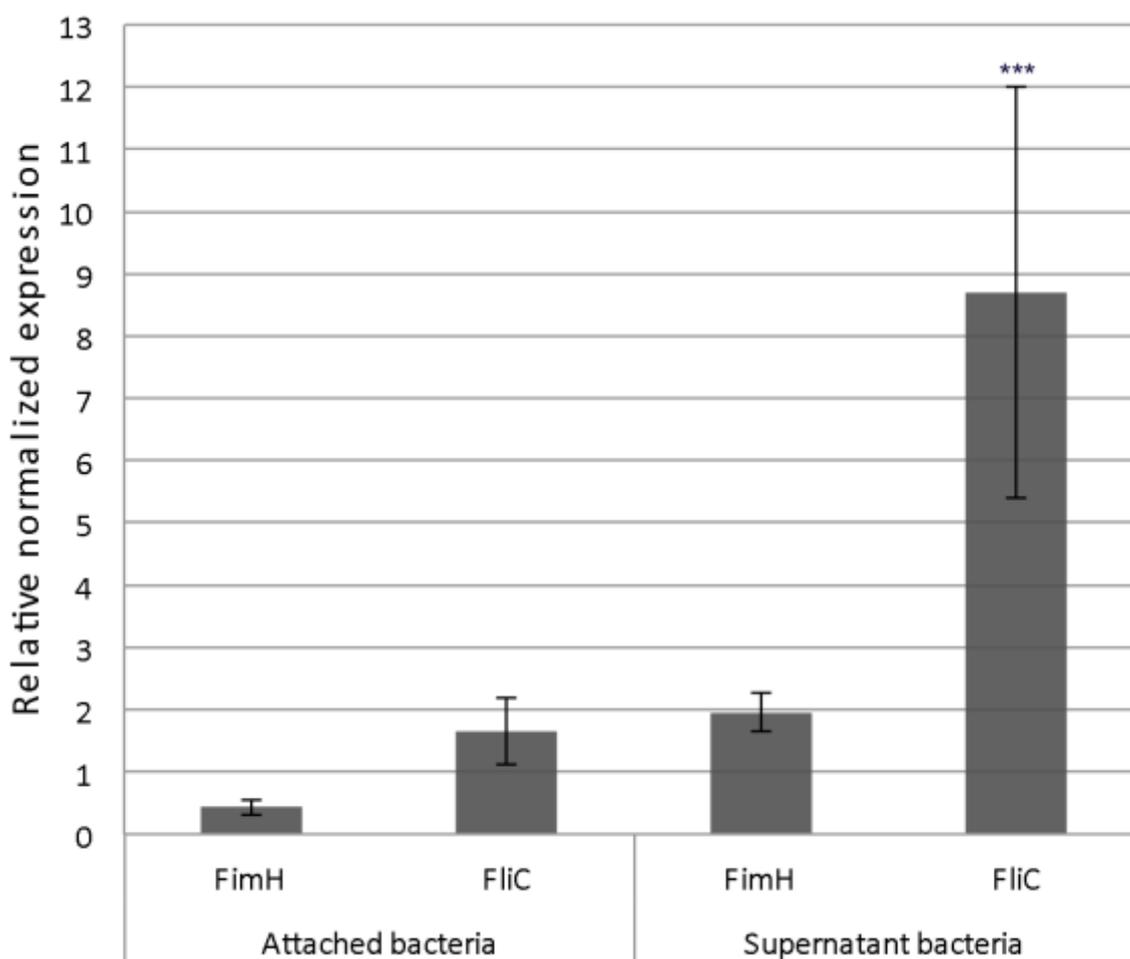
**Fig. 4:** Influence of different pre- and posttreatments of female Balb/c with OWE (750 mg/kg body weight) on the bacterial load in bladder and kidney after transurethral infection of the animals at day 0 with  $1 \times 10^8$  cells of UPEC strain CFT073. UC: untreated control; PC: positive control norfloxacin (100 mg/kg).

(A) 7-day pretreatment with OWA.

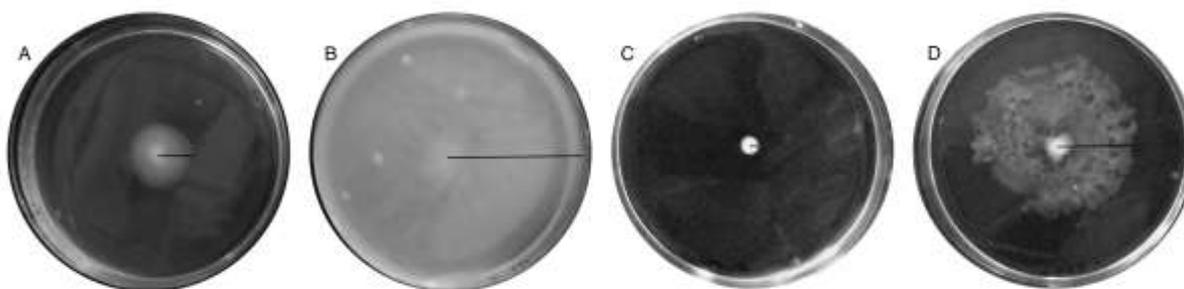
(B) 3-day posttreatment with OWA.

(C) 5-day posttreatment with OWA.

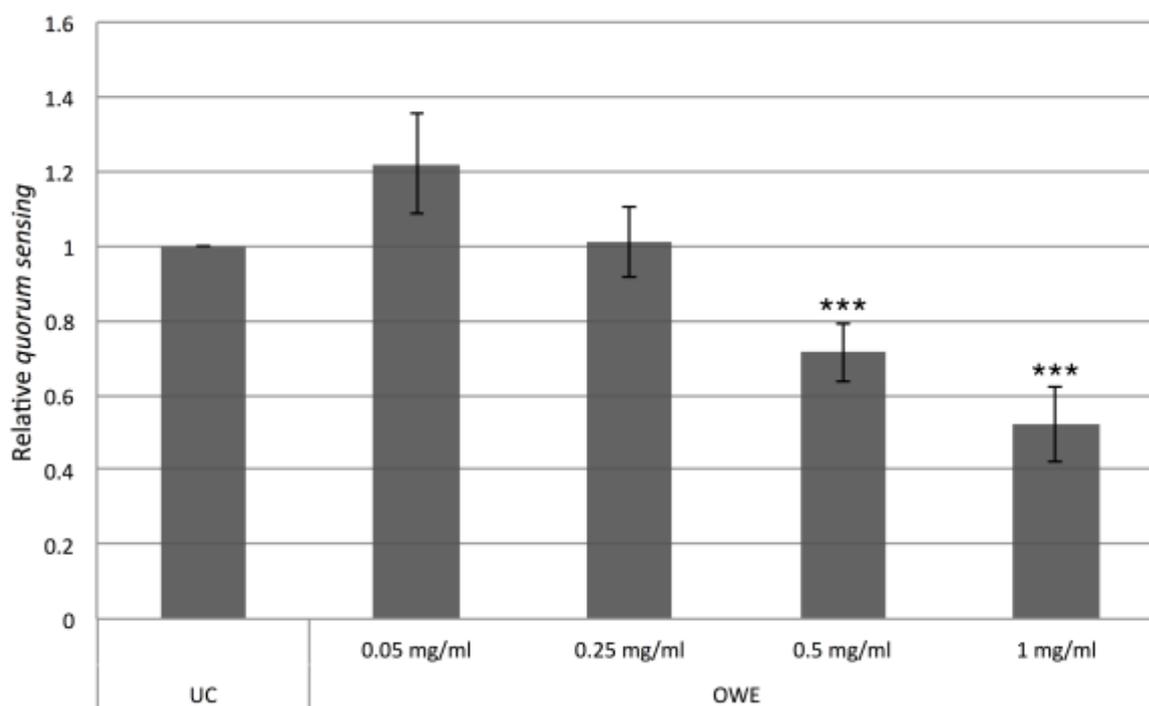
Animals were sacrificed 24 h after infection, complete bladder and kidneys were removed, homogenized and the bacterial load was determined by quantification of the colony forming units (CFU) after plating on LB agar. Each spot represents the CFU/bladder or CFU/kidney from one animal; bars represent the mean value. \*  $p < 0.1$ . \*\*  $p < 0.01$ .



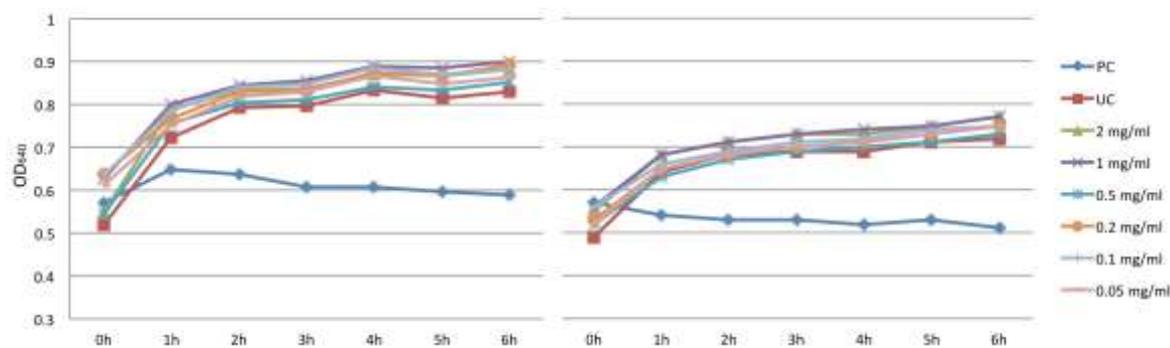
**Fig. 5:** Relative gene expression of selected virulence factors from *E. coli* strain NU14, pretreated with OWE (2 mg/ml) or in medium alone, as determined by q-PCR: comparison of the relative expression of *fimH* and *fliC* in bacteria incubated for 1 h together with T24 bladder cells (BCR 100:1) in host cell attached bacteria and free-floating bacteria in the supernatant. Expression levels are related to the untreated controls (= 1) and have been normalized to the expression of the endogenous 16S rRNA control gene. \*\*  $p < 0.01$ ; values represent the mean  $\pm$  SD of 3 independent assays with two technical replicates for each experiment.



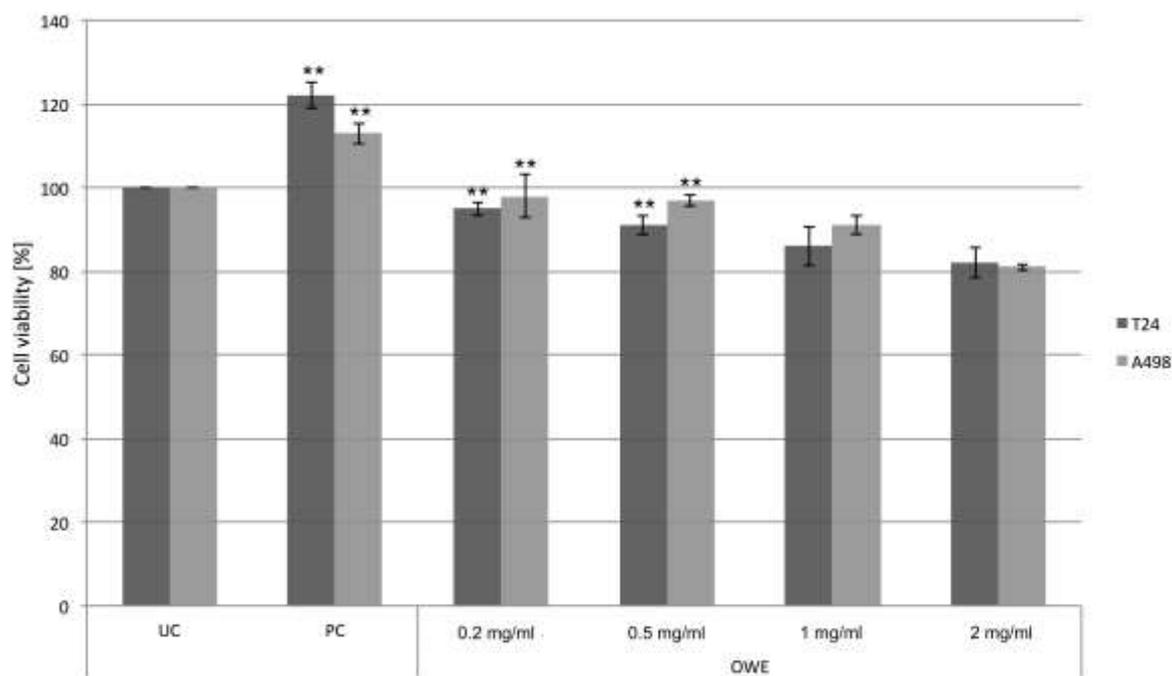
**Fig. 6:** Characterization of bacterial motility by swimming in soft agar. Cultures of the untreated control (A, C) and OWE (2 mg/ml) pretreated UPEC strain NU14 (B, D) were stabbed into 0.25% (A, B) and 0.5% soft agar plates (C, D) and incubated at 37 °C for 17 h. Bars in the respective images represent the rough diameter of the movement of the bacteria through the agar.



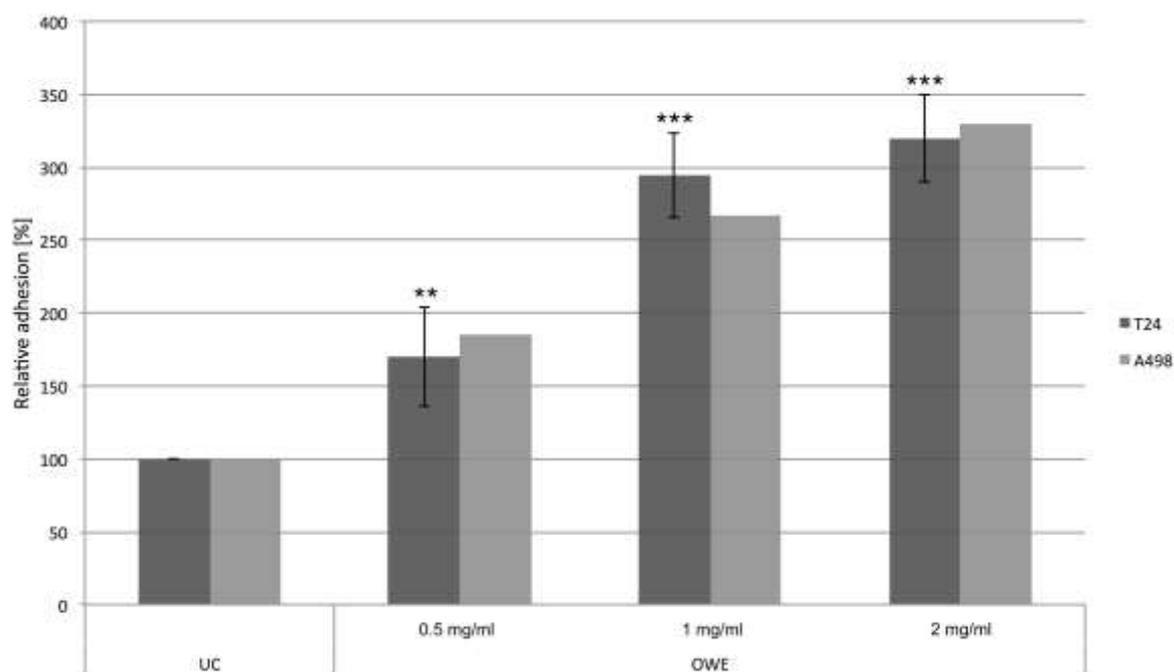
**Fig. 7:** Effect of OWE on the relative *quorum sensing* response of *E. coli* in relation to the untreated control (UC). Data represent the mean and  $\pm$  SD of three independent experiments with three biological replicates each; \*\*\*  $p < 0.001$ .



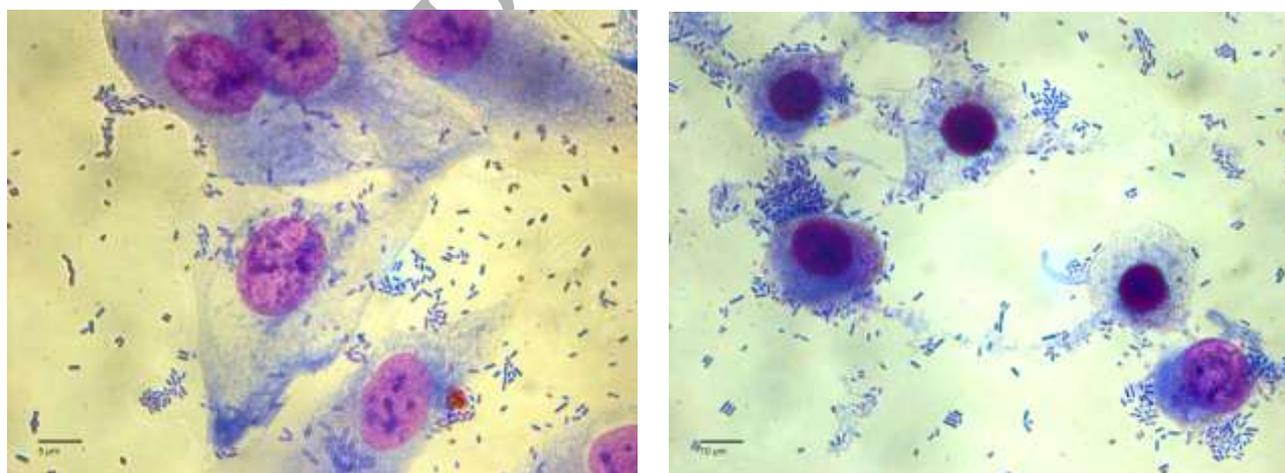
**Fig. S1:** Influence of OWE in different concentrations (0.05 to 2 mg/ml) on the OD of liquid cultures from UPEC strains NU14 (**A**) and *E. coli* 2980 (**B**) during 6 h incubation time. gentamycin (100  $\mu$ g/ml) served as positive control (PC). UC: untreated control.



**Fig. S2:** Influence of OWE at concentrations of 0.2 to 2 mg/ml on cell viability (MTT assay) of T24 bladder cells and A498 kidney cells after 24 h incubation time with the test extract. UC: untreated control; PC: positive control (10% FCS supplemented media). Bars represent  $\pm$  SD from 3 independent experiments with  $n = 6$  replicates.



**Fig. S3:** Influence of OWE at different concentrations after 90 min coincubation on the relative adhesion of FITC-labelled UPEC (strain NU14) to T24 bladder cells and A498 kidney cells within flow cytometric assay. UC: untreated control. Bars represent SD from 3 independent experiments with  $n = 2$  replicates.

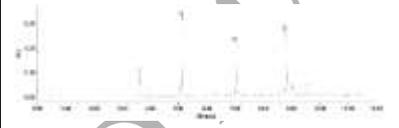


**Fig. S4:** Influence of OWE (2 mg/ml) after 90 min of coincubation on the adhesion of UPEC (strain NU14) to T24 bladder cells after Giemsa staining: (A) untreated control group (B) OWE-treated cells.

## Graphical abstract

**Aqueous extract from *Orthosiphon stamineus*  
leaves prevents bladder and kidney infection in mice**

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	<p style="text-align: center;"><b>Aqueous extract <i>Orthosiphon stamineus</i></b></p> 	<ul style="list-style-type: none"> <li>Antiinfective activity against UPEC in mice</li> <li>Antiadhesive activity against UPEC</li> <li>Downregulation of gene expression of <i>fimH</i></li> <li>Upregulation of gene expression of <i>flic</i></li> <li>Inhibition of <i>quorum sensing</i></li> </ul>
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**Table 1: Primer sequences for qRT-PCR of *fimH* or *fliC* gene transcription in UPEC. F: Forward primer; R: Reverse primer; Ref.: reference.**

Primer	Gene-ID (Ref.)	Sequence: 5'→ 3'
<i>fimH</i> -f <i>fimH</i> -r	3286745	CAATGGTACCGCAATCCCTA GCAGGCGCAAGGTTTACA
<i>fliC</i> -f <i>fliC</i> -r	26108594	ACAGCCTCTCGCTGATCACTCAA GCGCTGTTAATACGCAAGCCAGAA
16S rRNA-f 16S rRNA-r	85674274	GGCGCATACAAAGAGAAG ATGGAGTCGAGTTGCAGA

**Table 2: In vivo infection studies for investigation of potential anti-infective activity of OWE in BALB/c mice in different treatment cycles, at different doses and against different *E. coli* strains.**

Treatment before or after <i>E. coli</i> infection	Treatment period	Treatment groups, dose	<i>E. coli</i> strain	Monitoring of bacterial load in
Pre-treatment	4 days	Untreated control (water)	NU14	Bladder
		OWE 500 mg/kg/day	NU14	Bladder
		OWE 750 mg/kg/day	NU14	Bladder
	7 days	Untreated control (water)	NU14	Bladder
		OWE 750 mg/kg/day	NU14	Bladder
		Untreated control (water)	CFT073	Bladder
Post-treatment	3 days	OWE 750 mg/kg/day	CFT073	Bladder
		Untreated control (water)	CFT073	Bladder, kidney
		Norfloxacin 100 mg/kg/day	CFT073	Bladder, kidney
	5 days	OWE 750 mg/kg/day	CFT073	Bladder, kidney
		Untreated control (water)	CFT073	Bladder, kidney
		Norfloxacin 100 mg/kg/day	CFT073	Bladder, kidney
		OWE 750 mg/kg/day	CFT073	Bladder, kidney