

## **Research Article**

# **Seven-day oral intake of *Orthosiphon stamineus* leaves infusion exerts antiadhesive *ex vivo* activity against uropathogenic *E. coli* in urine samples #**

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# Dedicated to the 85<sup>th</sup> birthday of Prof. Gerhard Franz, University of Regensburg, Germany

## Abstract

*Orthosiphon stamineus* leaves (Java tea) extract is traditionally used for the treatment of urinary tract infections. According to recent *in vitro* data, animal infection studies, and transcriptomic investigations, polymethoxylated flavones from Java tea exert antiadhesive activity against uropathogenic *Escherichia coli* (UPEC). This antiadhesive activity has been shown to reduce bladder and kidney lesion in a mice infection model. As no data on antivirulent activity of Java tea intake on humans are available, a biomedical study was performed on 20 healthy volunteers who self-administered *Orthosiphon* infusion ( $4 \times 3$  g per day, orally) for 7 days. The herbal material used for the study confirmed to the specification of the European Pharmacopoeia, and UHPLC of the infusion proved rosmarinic acid, caffeic acid, and cichoric acid to be the main compounds beside polymethoxylated flavones. Rosmarinic acid was quantified in the tea preparations with  $243 \pm 22$   $\mu\text{g}/\text{mL}$ , indicating sufficient reproducibility of the preparation of the infusion. Urine samples were obtained during the biomedical study on day 1 (control urine, prior to Java tea intake), 3, 6 and 8. Antiadhesive activity of the urine samples was quantified by flowcytometric assay using pretreated UPEC NU14 and human T24 bladder cells. Pooled urine samples indicated significant inhibition of bacterial adhesion on day 3, 6 and 8. The urine samples had no influence on the invasion of UPEC into host cells. Bacterial proliferation was slightly reduced after 24 h incubation with the urine samples. Gene expression analysis (qPCR) revealed strong induction of fitness and motility gene *fliC* and downregulation of hemin uptake system *chuT*. These data correlate with previously reported datasets from *in vitro* transcriptomic analysis. Increased bacterial motility was monitored using a motility assay in soft agar with UPEC UTI89. The intake of Java tea application had no effect on the concentration of Tamm-Horsfall Protein in the urine samples. The present study explains the antiadhesive and anti-infective effect of the plant extract by triggering UPEC from a sessile life style into a motile bacterial form, with reduced adhesive capacity.

**Keywords:** *Orthosiphon stamineus*, Lamiaceae, adhesion, Java tea, Tamm-Horsfall Protein, uropathogenic *E. coli*.

**Abbreviations:**

FimH: adhesion of type 1 fimbriae, mannose sensitive; *fliC*: flagellum (H-antigen); HMPC: Herbal Medicinal Product Committee; IBC: intracellular bacterial communities; OWE: aqueous *Orthosiphon* extract; qPCR: quantitative real time polymerase chain reaction; UC: untreated control; 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 conditions, with an estimated global incidence of more than 150 million cases per year [1]. UTIs are caused, in about 80 % of all cases, by uropathogenic *Escherichia coli* (UPEC) but *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp. can also be involved in the pathogenesis of UTIs [2]. One of the first and most relevant steps in the pathogenesis of the infection is the specific recognition of the host cells of the lower urinary tract, followed by bacterial adhesion and subsequent invasion into the cell [3]. Therefore, the development of specific inhibitors of bacterial adhesion provides a new molecular target for combating UTIs. Antiadhesive entities act mainly at a very early stage of the infection and can also be involved in reducing potential reinfections. Subsequently, inhibition of host cell recognition and adhesion to the host cell membrane reduces the invasion of UPEC into the epithelial cells, the destruction of the epithelial cells and the formation of intracellular bacterial communities (IBC) by UPEC. IBC are, in many cases, responsible for the high degree of recurrence of infections [4–8]. Furthermore, inhibitors of UPEC invasion can reduce the intensity of infection [9]. The main adhesins of UPEC are mannose-sensitive type 1-pili (FimH, the most relevant adhesin in clinical isolates), and digalactoside-specific Pap-pili. FimH interacts with highly mannosylated uroplakins on umbrella cells of the host bladder and kidney cells. Additionally, many other pili contribute to bacterial adhesion. Afimbrial Afa/Dr adhesins bind to type IV collagen [10]. Interaction of UPEC with DAF proteins (human decay-accelerating factor) is fundamental for the internalization of the bacteria into intracellular vacuoles [11]. Additionally, amyloid-like curli interact with proteins of the extracellular matrix, leading to enhanced adhesion of UPEC to the host cells [12].

A variety of traditional herbal remedies have been investigated against UTI focusing on the elucidation of the underlying mode of action. Besides antibacterial compounds (e.g. arbutin, isothiocyanates), anti-inflammatory extracts (e.g. *Ononis spinosa* L. roots [13], *Matricaria recutita* L. flowers, *Achillea millefolium* L. flowers, inhibitors of human hyaluronidase-1 (e.g. clitorienolacton B from *O. spinosa* roots [13, 14], inductors of Tamm-Horsfall protein as part of the innate immune defense of the renal system (e.g. *Vaccinium macrocarpon* Aiton fruits [15]), also anti-adhesive and anti-invasive extracts and compounds have been identified (e.g. phthalides from *Apium graveolens* L. fruits [16], flavones and flavonols from *V. macrocarpon*

fruits [17], flavan-4-ol derivatives from *Zea mays* L. stigmata [5], polymethoxylated flavones from *Orthosiphon stamineus* leaves [18]).

In rational phytotherapy extracts from the leaves of *O. stamineus* BENTH. (syn. *O. aristatus* MIQ., Java tea) from the plant family of Lamiaceae are widely used for UTI. The plant originates from the tropical Asia and is mainly cultivated in Indonesia. Tea preparation form *O. stamineus* are widely used in the tropical areas and Java tea is popularly known also as “Kumis Kucing” in Indonesia and “Miasai Kucing” in Malaysia. The herbal material used in the Western countries is mainly imported from Indonesia and freshly brewed Java tea is widely used as refreshing tea in the food sector, but is also known as herbal remedy for medical use and for treatment and prophylaxis of UTI. This medical use is supported by monographs of competent authorities such as the Herbal Medicinal Product Committee (HMPC) of the European Medicines Agency [19], or other scientific bodies such as the European Scientific Cooperative on Phytotherapy [20]. The traditional use is described in the recommendations of HMPC, where the use of aqueous (mainly) and hydroalcoholic extracts (20 to 60 % EtOH content) are both accepted for medical registration in the EU.

The phytochemical composition of *O. stamineus* leaves has been investigated extensively (for review see [21]).

Within a systematically investigation on *O. stamineus* for treatment of UTI an aqueous extract with the designation OWE (*Orthosiphon* water extract) has been prepared by hot water (yield 20 % w/w, related to the dried starting material; herbal material/extract ratio = 1 : 5) and analytical quantification of the extract by ICH2 guideline validated HPLC revealed a content of the three analytical marker compounds of OWE as follows: caffeic acid  $7.1 \pm 0.3$  mg/g, cichoric acid  $7.9 \pm 0.4$  mg/g and rosmarinic acid  $10.9 \pm 0.4$  mg/g [8]. Detailed description of the manufacture of OWE and the analytical protocols, including UPLC chromatograms is displayed in [8].

*In vitro* testing of the OWE indicated concentration-dependent and significant antiadhesive effects against UPEC [8]. OWE showed no direct cytotoxicity in UPEC as bacterial proliferation was not influenced by OWE. In addition, cellular vitality and mitochondrial activity of human bladder and kidney cells was not negatively influenced by OWE [8]. Detailed investigation and bioassay-guided fractionation of OWE identified polymethoxylated flavones as compounds which are responsible for the observed antiadhesive effect [18]. This seems interesting, as it is known from literature that polymethoxylated flavones, such as sinensitin,

nobiletin, eupatorin or tangeretin are bioavailable in rats after oral application and metabolites of these flavones as the respective glucuronides, partially demethylated flavones and sulfates can be detected in urine samples [22–24]. OWE demonstrated strong influence on bacterial *quorum sensing* and significantly reduced the gene expression of *fimH* under *in vitro* conditions [8]. OWE increased the expression of the motility/fitness gene *fliC*, which promoted a change of phenotypes towards an increased bacterial motility. These effects have additionally been investigated in detail by transcriptome analysis of OWE-treated UPEC, which indicated that the reduced bacterial adhesion is due to a decreased formation of the bacterial fimbriae due to disturbance of the chaperon-usher system [21]. Anti-adhesion can cause a relevant anti-infective effect, as has been shown in a mice infection model with animals orally treated with OWE, leading to significant reduced infection of bladder and kidneys [8]. *In vivo* animal studies (mouse infection model) showed that OWE has significant anti-infective effects (750 mg OWE/kg, p.o. treatment of the animals for 3 and 5 days) and lowered the bacterial colonization in the kidneys and bladder after transurethral infection with UPEC strain CFT073 [8]. Furthermore, 4- and 7-day pre-treatment of the mice with OWE prior to infection with UPEC NU14 reduced the colonization of the bladder [8]. From the published data described above, OWE seems to be a potent antiadhesive agent. On the other hand, it is important to recognize that results obtained from *in vitro* or other preclinical experiments cannot be directly translated into clinical results [19]. The use of biomedical studies with volunteers evaluating kinetic aspects of the orally administered remedy could help closing the gap between preclinical studies and clinical investigations. In addition to that, monitoring potential functionality by *ex vivo* investigations, could help clarifying the bioavailability of active compounds after oral ingestion.

For this the present study describes the results of a biomedical study during which *Orthosiphon* herbal tea was used, according the recommendation of the HMPC. Urine samples, obtained from volunteers were to be investigated within the present study on potential antiadhesive effects against UPEC in order to clarify the occurrence of antivirulence effects.

## Results and Discussion

The influence of a 7-day oral intake of aqueous infusions prepared from *O. stamineus* leaves was investigated by a biomedical study. The herbal material used for tea preparation conforms to the specification of European Pharmacopoeia for *O. stamineus* leaves [25]. Preparation of the *O. stamineus* leave infusion was performed using 3.0 g herbal material in a standard cellulose tea bag, addition of 200 mL of boiling water, and 10 min extraction time. Analytical

investigation of these Java tea infusions revealed a dry yield of 0.91 g, corresponding to 30.3 % (w/w), related to the herbal material (herbal substance:extract ratio = 3:1) after lyophilisation. A typical U(H)PLC chromatogram obtained from the lyophilized extract, obtained from an exemplary herbal infusion is displayed in the Supplementary Data (Figure 1S), indicating a qualitatively very similar profile compared to the hot water extract OWE, described in previous investigations in the literature [8, 18, 21]. Main compounds were - as expected - rosmarinic acid, cichoric acid, caffeic acid and minor amounts of polymethoxylated flavones. To investigate the reproducibility of the tea preparation prior to the biomedical study, 9 randomly selected subjects not involved in the study were asked to prepare a java tea. They were instructed to do so according to the same protocol as the study participants (extraction of a tea bag containing 3.0 g of Java tea with 200 mL of boiling water in a tea cup for 10 min, moving the tea bag every 2 to 3 min and squeezing it after 10 min).

The tea samples prepared in this way were analyzed by U(H)PLC [8] for content of rosmarinic acid against the respective reference standard after calibration. Average content of rosmarinic acid was calculated with  $243.3 \pm 21.5 \mu\text{g/mL}$ . From this a relative standard deviation of  $\pm 9\%$  was calculated, indicating sufficient reproducibility and validity of the study product.

From the previously quantified content of rosmarinic acid in Java tea (1.8 %, c.f. Material and Methods), an amount of 54 mg per tea bag (3 g) can be calculated. The quantified content per serving (ca. 50 mg in 200 mL infusion) fits this very well.

For the biomedical study, Java tea infusions from 3 g of the herbal material were self-administered four times a day. This dosage is based on the recommendation of the HMPC for *Orthosiphon* tea [19]. The study primarily aimed at investigating the potential antiadhesive capacity of the urine samples against UPEC and, in addition, the influence of the urine samples on UPEC invasion into T24 bladder cells, UPEC proliferation, gene expression and potential changes in the UPEC phenotype. After acceptance of the study by the ethic commission twenty-two volunteers in the U.K. (average age 26.7 years, 12 males, 10 females) were enrolled in the study. After collection of urine samples at days 1, 3, 6 and 8, the urine of two participants was excluded due to a non-continuous intake of the tea preparation. Within the scope of the 7-day consumption of *Orthosiphon* tea, no intolerances were observed.

The monitoring of morning urine samples obtained from the volunteers before (day 1) and during the consumption (day 3, 6, 8) of Java tea showed no abnormalities concerning the standard urine parameters, with the exception that the potassium concentration tended to

decrease over the study time. 33 % (day 6) of the urine samples had a relatively high osmolarity at the upper limit ( $> 700$  mosmol/L) of the reference standard range (50 to 1200 mosmol/L). This seems to be due to nutritional habits of the volunteers with either a low water intake or a high daily salt consumption. The high osmolarity affected the outcome of the subsequent functional testing: during the 30 min incubation of host cells together with the urine samples ( $> 700$  mosmol/L) drastic morphological changes of the T24 bladder were observed, possibly due to osmotic stress. Consequently, investigation of the urine samples for potential antiadhesive activity could not be performed by co-incubation of the host cells together with UPEC and urine samples. Instead a 2 h pre-incubation of the bacteria with the urine samples was performed. Subsequently, the urine was removed after the preincubation of UPEC by a washing step. The pre-treated bacteria were added to the T24 bladder cells and the mixture was incubated for one hour. Evaluation of the adhesion of the fluorescent-labelled bacteria was performed by flow cytometry and the relative adhesion was calculated for every individual against the day 1 control urine.

The analysis of the individual urine samples was performed by three independent assays with  $n \geq 2$  technical replicates. The individual relative adhesion values showed a homogenous distribution (Figure 1A) with the exception of two data points, which were statistically investigated by Grubbs' test for outliers. After elimination of the two data points, on day 3 and 6, a significant reduction of the relative adhesion on day 3 (mean adhesion 80 %) and day 8 (mean 83 %) was observed (Figure 1B); adhesion values on day 6 were reduced (mean 92 %) as well, but not significantly when compared to the respective control values from day 1. Investigation of the pooled urine samples indicated significant inhibition of bacterial adhesion to T24 bladder cells on day 3, day 6 and day 8 (Figure 1C).

Evaluation of the data concerning a potential influence of ethnicity on the outcome of the adhesion assay indicated no significant differences between Caucasian and Asian participants (data not shown).

Subgroup analysis for investigation on the potential influence of the volunteers' sex indicated moderate benefits of *Orthosiphon* application for male individuals with significantly reduced bacterial adhesion on day 3 and day 8 (Figure 1D). This effect was more pronounced than that obtained for female participants. Nevertheless, these correlations need to be interpreted carefully, as the evaluated data originated from a limited number of samples.

The results of this preliminary study show that a 7-day oral administration of *Orthosiphon* tea evokes a significant antiadhesive effect of urine and limits the interaction of UPEC with bladder

cells. Based on these results, it can further be inferred that compounds contained in the OWE are systemically bioavailable after oral intake and that active metabolites are eliminated through the urinary system. It should be pointed out that antiadhesion can cause a relevant anti-infective effect, as has been shown in a mice infection model with animals orally treated with *Orthosiphon* aqueous extract, leading to significant reduced infection of bladder and kidneys [8].

To exclude that the observed antiadhesive effect was due to antiproliferative or direct cytotoxicity of the urine samples against UPEC, the pooled urine samples from day 1, 3, 6 and 8 were tested over 24 h for any direct effect on *in vitro* proliferation of UPEC NU14 (Figure 2). UPEC grown in day 1 urine samples showed a slightly higher proliferation rate compared to the samples grown in day 3, 6 and 8 urine, but interestingly the differences after 24 h of incubation were negligible. To assure that the results obtained within the adhesion assay were not hampered by such slight antiproliferative effects, a short time study over 2 h incubation of the fluorescent-labelled bacteria in day 1, 3, 6, 8 urine samples was performed. No differences were found in all test groups (data not shown). This data suggest that urine samples had no relevant antiproliferative effects which would have influenced the adhesion data.

While bacterial adhesion to host cells is the initial step of the pathogen-host interaction, for successful infection the attached UPEC need to be internalized by membrane fusion into the cell. As some reports are available on natural products interfering with bacterial invasion into the host cell [9], a specific invasion assay was performed with UPEC NU14, which had been pre-incubated for 2 h with the pooled urine samples (day 1, 3, 6, 8). After removal of the urine, the pre-treated bacteria were incubated together with T24 cell. Non-invaded bacteria were eliminated by gentamycin. T24 cells were lysed and aliquots of the cell lysate were plated onto agar. Colony forming units were quantified after 24 h. A slight, non-significant reduction in intracellular bacteria count was recorded, unfortunately, with a high standard deviation, due to the complexity of the assay (Figure 3). Comparison of these results with the data obtained from the adhesion assay did not reveal relevant differences (Figure 3).

From these data, it can be concluded that the reduction in the number of intracellular bacteria is not due to an anti-invasive effect, but is mainly caused by inhibition of the bacterial adhesion to the host cell.

*In vitro* studies with aqueous *Orthosiphon* extract on the gene expression of UPEC indicated significant downregulation of bacterial adhesins (curli, type 1-, F1C-, and P-fimbriae and of the chaperone-mediated protein folding/unfolding and pilus assembly) [21]. In contrast, flagellar and motility-related genes had been shown to get upregulated [21]. As gene expression,

measured under *in vitro* conditions by use of a crude extract in contact with the bacteria may be different compared to the *in vivo* situation, similar qPCR analysis experiments were performed *ex vivo* by using the pooled day 8 urine samples, which were obtained from the *Orthosiphon*-treated volunteers. After 2 h incubation time of UPEC NU14 with the respective urine samples the following genes were monitored by qPCR: *fimH*, *fimC* and *fimD* (type 1-fimbriae), *csgA* (thin aggregative fimbriae-curli), *papGIII*, *papC*, *papD*, *prsGIII* (P-fimbriae), *sfaG* (S-fimbriae), *focG* (F1C-fimbriae), *fyuA* (*Yersiniabactin* siderophore), *chuT* (hemin uptake system), *fliC* (flagellum H-antigene, motility and fitness). Significant upregulation was observed for the motility and fitness gene *fliC* (Figure 4), in accordance to recent data described for *Orthosiphon*'s *in vitro* studies [21].

Day 8 urine pre-treatment resulted in about 40 % downregulation of the *chuT* transcript, responsible for the formation of proteins responsible for iron uptake. This downregulation is in accordance with data reported recently for the extract under *in vitro* conditions [21]. No other genes showed relevant or significant changes. Interestingly, no changes in the expression of genes responsible for attachment was observed. This is contrast to the reported *in vitro* data, which showed strong downregulation of type 1 fimbriae associated genes as a consequence of contact between *Orthosiphon* extract and UPEC [8, 21].

It could well be that under *in vitro* condition quite high concentrations of the extract (2 mg/mL) will influence the chaperon usher mediated formation of pili, while the effect is not occurring after incubation with the *ex vivo* urine samples, because of much lower concentrations of the respective metabolites.

In order to investigate whether this effect of the urine samples on gene expression level is further reflected by changes in bacterial phenotype, a motility assay was performed, which is strongly related to the activity of the flagellin FliC [8, 26]. Bacteria were pre-incubated for 2 h in pooled urine samples (day 1, 3, 6, and 8) and stabbed on semi-solid agar. It is known that bacteria in semi-solid agar solutions will not only disperse 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; increased fitness and motility of the bacteria will thereby result in different spreading behaviour in the agar [8, 27]. As an increased motility of UPEC is a disadvantage for an adequate adhesion, the increased expression of *fliC*, leading to a high motility phenotype appears as a cause for reduced bacterial adhesion [28, 29]. Day 3, 6, and 8 urine treated bacteria showed higher motility compared to the untreated bacteria (Figure 5). The respective data cannot be considered significant due to the high standard deviation, but a clear tendency for

higher motility can be observed in the individual experiments. Thus, data obtained from the gene expression analysis, indicating increased *fliC* expression, can be correlated to the changed phenotypic behaviour.

Finally, the influence of the *Orthosiphon* application on the concentration of Tamm-Horsfall Protein (THP, syn. uromodulin) in the urine samples was investigated. THP is known to be part of the innate immune defence and is produced exclusively by the renal tubular cells in the Henle loop of the kidney [30, 31].

It is known that THP secretion in the urine can be stimulated by exogenous noxes, and especially extract of cranberry fruit (*Vaccinium macrocarpon*) have been shown to be strong inducers of THP formation, which subsequently leads to significantly elevated anti-adhesive activity of urine against UPEC [15]. Therefore, all urine samples were investigated using a specific ELISA for its THP concentration (Figure 6). No significant changes between day 1, 3, 6, and 8 urine were recorded. As THP formation in the kidney is also gender-specific, subgroup analysis for male and female participants was performed, but no relevant difference was found. From this point of view, *Orthosiphon* infusion has no influence on THP formation in the kidney.

Summarizing, the present study clearly proves strong and significant antiadhesive and antivirulence effects of Java tea in urine samples after oral administration. Direct cytotoxic effects against UPEC can be excluded, but a strong influence in the pathogen-host interaction is obvious. The clinical data obtained within the present study are in good accordance with previously reported data obtained by *in vitro* experiments and animal infection studies. It seems promising that under *in vivo* conditions antiadhesive and antivirulence effects are observed in the urine samples, indicating that the compounds from *O. stamineus* leaves, being responsible for the observed effects are bioavailable after oral ingestion. Additionally, the respective metabolites are obviously excreted in the urine. Based on the recent *in vitro* studies polymethoxylated flavones have been claimed to be the main antiadhesive compounds in Java tea extracts [18]. In particular, flavones with high lipophilicity due to multiple methoxylations in the A- and B-rings (e.g. sinensetin, ladanein, 5,6,7,4'-tetra-methoxyflavone, 5-hydroxy-6,7,3',4'-tetra-methoxyflavone) exert high anti-adhesive activity. Polymethoxylated flavones (e.g. sinensitin, eupatorin) have been shown to be bioavailable after oral administration to rats and partially demethoxylated metabolites have been identified in rat urine samples [23, 24]. From this point of view the fraction of polymethoxylated flavones from Java tea infusion is

assessed to be responsible for the observed antiadhesive effects found for the urine samples in the above described biomedical study. Unfortunately, we had been not able to quantify the amount of these compounds in the urine samples, despite the use of sensitive and specific LC-MS protocols (data not shown). This is due to the high variability of different polymethoxylated flavones in Java tea (> 6 different aglycones have been described for *O. stamineus* leaves by [18]) and subsequent metabolic demethylation will additionally lead to the formation of even more products, excreted also in the urine. This complex mixture has not been accessible to our LC-MS analytical protocols. On the other side there is no doubt on the antiadhesive functionality of the investigated urine samples against UPEC. The underlying mechanism still seems to be not clear. A stimulation of host innate immune defense via THP-induction, similar to the mode of action described for Cranberry extracts for UTI [15] can be excluded. Also, direct inhibition of the bacterial adhesins (e.g. FimH) by Java tea metabolites is unlikely, based on the findings that the expression of relevant fimbrial genes is not influenced by the urine samples and also FimH-mediated yeast agglutination is not reduced by the test samples. On the other side it is interesting that the motility gene *fliC* is significantly upregulated, which is also reflected by increase motility in the respective UPEC phenotypic. From this point of view, it can be assumed that *Orthosiphon* triggers the bacterial cell into a high motile form, which again leads to a strongly reduced sessile form and bacterial adhesion to the host cells. Motility and adhesion are in general two different living forms, being contrary to each other: reduced adhesion during high motility phase or increase attachment of UPEC to host cells in cases were the flagella activity is downregulated. As these *Orthosiphon* effects have been shown in the present study by the urine samples and also in a recent report by an *in vitro* transcriptomic study [21] the assumption that *Orthosiphon* triggers the UPEC life style from a more sessil into a motile form has been confirmed. Which molecular factors are responsible for this switch remains unclear at the moment and detailed molecular studies on the influence of polymethoxylated flavones on the flagella regulation are required to pinpoint the exact molecular mechanism.

## Materials and Methods

### *General experimentation procedures, solvents, reagents*

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* (*Orthosiphon folium*), batch no 201808131005, was obtained by Martin Bauer Group. The material was identified by AH and complied with the specifications of the European Pharmacopoeia [25] (identity TLC: conforms, loss on drying: conforms, foreign matters: conforms, total ash: conforms, content rosmarinic acid HPLC 1.8 % w/w): conforms, pesticides: complies to EU396/2005, Ph. Eur. 2.8.13). Additionally, identity testing for polymethoxylated flavones, caffeic acid, and cichoric acid was performed by U(H)PLC as described recently [8]. A voucher specimen of the material is retained in the archives of Institute of Pharmaceutical Biology and Phytochemistry, Münster, Germany under the designation IPBP 495.

Manufacture of the study teas for the biomedical study was performed in a community pharmacy store under controlled conditions complying with GMP. 3.0 g of *Orthosiphon* leaves were filled into tea filter bags (Caesar & Loretz). Each tea bag was labelled with the relevant information for every volunteer.

Quantitation of rosmarinic acid in Java tea infusions was performed by use of rosmarinic acid reference compound (Sigma-Aldrich, content 97.0 % HPLC) in the range of 50 to 530 µg/mL.

#### Biomedical study design

The study protocol “An intervention with Java tea (*Orthosiphon stamineus*) – Metabolite changes in urine” was approved (March 20 2019) by the UCL Research Ethics Committee, London, U.K. (project: 5101/001).

Included were healthy men and women with an age of 18 to 40 years. Exclusion criteria is listed in the supplementary material.

Twelve male and ten female participants gave written informed consent to participate in the study. The mean age was 26.7 years, median 25.5 years, ranging from 21 to 35 years. Ethnicity: 50% were Caucasian (11/22), 41% Asian (9/22), 2 participants (9 %) mixed ethnicity. Preferred diet of the volunteers was documented by the questionnaires. Normal balanced diet was recorded for 82 %, vegetarian and pescatarian diets were stated for one participant each, and 14 % reported to have an unbalanced, carbohydrate-loaded diet. Concerning the potential intake of drugs during the biomedical study 9 % of the volunteer’s reported on the intake of hormone-based contraceptives at the time of the study, while the rest of the volunteers reported no use of concomitant medications. All participants volunteered to take *Orthosiphon* tea (4 × daily, 3 g per dosing) over a 7 days period (one cup in the morning, one at noon, one in the afternoon and

one in the evening), and to collect a sample of their first morning urine of the day on day 1 (prior to *Orthosiphon* tea application = control urine), 3, 6, and 8 for *ex vivo* studies.

Before starting the trial, volunteers were instructed to abstain from consumption of any other products containing cranberry or phytochemically or botanically similar fruits (especially from the plant family Ericaceae) two weeks before and during the study. Each participant was asked to drink a cup of freshly prepared *Orthosiphon* tea, made from prepacked tea sachets, each containing 3.0 g of the herbal material, four times a day, reasonably distributed through the morning, afternoon and evening, regardless of food. The daily intake was equivalent to 12.0 g herbal material per day for 7 days, based on the recommendation of the Committee on Herbal Medicinal Products [19]. Preparation of the Java tea infusion was performed by infusing one filter tea bag for 10 min in 200 mL of boiling water. The addition of sugar, honey or lemon juice after preparation wash allowed. The tea could be taken independently of meals, but the volunteers were instructed to take it every day approximately at the same time (+/- 2 hours) and to tick the box of the corresponding scheduled intake on the detailed calendar provided.

Generally, the first midstream urine of the day was collected and used for functional and analytical investigations. A control urine sample (day 1) was collected prior to the consumption of the tea.

Volunteers were instructed to collect the samples and store them in a refrigerator (+2 to 8 °C), or, if possible, in a freezer (< -5 °C), until handing it over to the research facility, the very same morning of collection. The urine was finally stored in a – 20 °C freezer until analysis.

By the end of the study, two drop-outs (one male, one female) were identified and removed from the data collection, due to non-continuous intake of infusions; one participant did not collect day 8 urine. Samples of day 1, 3 and 6 of the participant were included. Therefore, 79 urine samples from 20 volunteers were included in the subsequent *ex vivo* evaluation.

#### *Urine samples preparation and analysis*

The urine samples were stored at -20 °C until use. 2 mL of each urine sample from all volunteers were pooled and named day 1 PU (pooled urine), day 3 PU, day 6 PU, day 8 PU.

All urine samples obtained were tested for their pH, density, and content of creatinine, leukocytes, erythrocytes, sodium, potassium, chloride, bilirubin, urobilinogen, glucose, nitrite, protein, ketones (Supplementary Data, Table 1S and Table 2S). Urine osmolality was determined after dilution with highly purified water (Millipore quality) using a Semi-Micro Osmometer K-7400 (Knauer) (Supplementary Data, Table 3S)

## *Cell culture and microbiology*

### *T24 cells cell line and growth conditions*

T24 cells (ATCC HTB-4) represent a human epithelial bladder cell line, derived from the bladder carcinoma of an 82 years old Swedish female [32]. These cells have been demonstrated suitable for adhesion and invasion *in vitro* assays with UPEC [33] and were kindly provided by Prof. Straube (University of Jena, Germany). Cultivation was performed as described by [18]. The cultivation of the cells was performed in Dulbecco's Modified Eagle Medium with high glucose and L-glutamine (DMEM) (Biochrom), supplemented with 10 % FCS (Biochrom) and 0.5% penicillin/streptomycin (Biochrom) at 37 °C and 5% CO<sub>2</sub>.

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

Bacterial strains: UPEC strain NU14 (NCBI *txid569579*), a fully sequenced clinical isolate, obtained from a patient with an acute cystitis, was provided by Prof. Dr. U. Dobrindt, University of Münster, Germany [34], as well as the clinical cystitis isolate UPEC UTI89, a clinical cystitis isolate (NCBI: *txid364106*) was provided by Prof. Dr. U. Dobrindt, University of Münster, Germany [35].

Optical density (OD) was determined at  $\lambda = 640$  nm and  $1 \times 10^9$  colony forming units was equivalent to an OD<sub>640</sub> of 5.0.

Bacteria from frozen stocks were cultivated for 48 h on UPEC agar (Agar-Agar 15 g, Bacto Tryptone 10 g, NaCl 8 g, glucose 1 g, yeast extract 1 g, CaCl<sub>2</sub> 2 g, purified water 1 L) were used. CaCl<sub>2</sub> supplementation is supposed to increase the type 1 fimbria expression [36].

Urine culture: 1 CFU of overnight agar grown bacteria was transferred to 10 mL in 50 mL tubes and incubated at 37 °C overnight (~15 h) in a steady culture at 37 °C/5 % CO<sub>2</sub>.

*Monitoring of bacterial growth in liquid culture and adhesion assay by quantitative flow cytometry* was performed as described by [5, 15, 17].

### *Monitoring of bacterial growth in urine*

Pooled urine samples from day 1 (control urine), 3, 6, and 8 were transferred in aliquots of 180  $\mu$ L each into a 96 well plate. Additionally, pooled urine supplemented with gentamycin (100  $\mu$ g/mL) was used as positive control. Overnight agar grown bacteria (UPEC NU14) were harvested and suspended in UPEC liquid medium. The OD<sub>640nm</sub> was adjusted to 0.5. 20  $\mu$ L of the suspension was added to the urine samples and gently mixed. The plate was incubated at 37 °C and bacterial growth was monitored by measuring the optical density every 60 min over a 6 h period and after 24 h at  $\lambda = 640$  nm. Moreover, the OD of the urine samples without any additional bacteria was determined in order to ascertain that there are no differences in the OD due to different coloured urine.

#### Adhesion assay with urine samples by quantitative flow cytometry

In general, FITC-labelling of UPEC and flow cytometric adhesion assay was performed as described by [4, 37]. Agar grown UPEC NU14 were labelled under light protection with fluorescein isothiocyanate (FITC) as follows: UPEC were resuspended in 1 mL sterile saline solution (NaCl 150 mM, Na<sub>2</sub>CO<sub>3</sub> 100 mM, pH 8.0).  $14 \times 10^8$  bacteria were resuspended in 900  $\mu$ L of saline solution. 100  $\mu$ L of a FITC solution (10 % in DMSO) were added and incubated for 60 min at 37 °C in a thermomixer (Eppendorf) at 300 rpm. The labelling process was terminated by pelleting the bacteria ( $10.000 \times g$ , 5 min). Labelled bacteria were washed 3  $\times$  with 1 mL PBS to remove excess FITC, resuspended in 1 mL PBS and adjusted to OD<sub>640 nm</sub> of 4 (determined from a 1: 20 dilution). All further steps with FITC-labelled *E. coli* were carried out under direct light protection. For sample preparation 900  $\mu$ L of individual or pooled urine samples were mixed with 100  $\mu$ L of FITC-labelled bacteria suspension (OD<sub>640 nm</sub> 0.4) and incubated for 2 h at 37 °C/ 5% CO<sub>2</sub>. Subsequently the suspensions were centrifuged ( $10.000 \times g$ , 5 min) and pelleted bacteria washed twice with 1 mL PBS each. Washed bacteria were resuspended in 1 mL DMEM and added to the prior prepared T24 cells. T24 cells ( $1.25 \times 10^5$  cells/well) were seeded into 6-well plates and incubated at 37 °C/5% CO<sub>2</sub> until 90 % confluence was reached (corresponding to 800.000 cells, after approximately 48 h of incubation). The medium was removed and cells were washed twice with PBS (1 mL) and once with DMEM (1 mL). For adhesion experiments, the bacteria to cell ratio (BCR) of 100:1 was used. UPEC and T24 cells were incubated for 1 h at 37 °C. Subsequently, unattached UPEC were removed by gently washing the cells 3  $\times$  with 1 mL PBS/well. Cells were detached by addition of 1mL trypsin/EDTA for 4 min at 37 °C. Trypsinisation was stopped by addition of 2.5 mL DMEM. The content of each well was transferred to tubes and centrifuged for 5 min at  $450 \times g$ . The supernatant was discarded, and the cells resuspended in 700  $\mu$ L of DMEM.

Fluorescence of the cell suspension was measured by flow cytometry (FACS Calibur, Software: BD CellQuest Pro V 5.2, BD Biosciences). For data evaluation, 10.000 counts per sample were used. Day 1 urine samples were used as untreated control urine while day 1 urine added with antiadhesive hydroalcoholic *Zea mays* extract [5] (1 mg/mL) served as positive control.

#### *Invasion assay with urine samples [38]*

Cells ( $1.25 \times 10^5$  cells/well) were seeded into 6-well plates and incubated at 37 °C / 5 % CO<sub>2</sub> until 90 % confluence was reached (corresponding to 800.000 cells, after approx. 48 h of incubation). After this incubation T24 cell culture medium was removed, cells were washed twice with PBS and once with DMEM. Agar grown UPEC NU14 were harvested and suspended in 1 mL DMEM. The OD<sub>640 nm</sub> was adjusted to 4. 100 µL of the suspension were added to 900 µL of each urine sample and incubated for 2 h at 37 °C/5 % CO<sub>2</sub>. Subsequently the suspensions were centrifuged (10.000 × g, 5 min) and pelleted bacteria washed twice with 1 mL PBS each. Washed bacteria were resuspended in 1 mL DMEM and added to the prior prepared T24 cells. Bacteria and T24 cells were incubated for 1 h at 37 °C/5 % CO<sub>2</sub>. Bacteria which did not interact during the incubation with T24 cells were removed by 3 times washing the T24 cells with 1 mL PBS/well. Subsequently DMEM containing 100 µg/mL of the membrane-impermeable antibiotic gentamycin was added for 1 h at 37 °C to the samples in order to eliminate selectively extracellular bacteria. The antibiotic was removed by rinsing three times with PBS. Finally, cells were lysed (15 min, room temperature) by addition of 0.1 % Triton X-100, the lysate was plated in 1:250 dilution onto UPEC agar and incubated for 24 h at 37 °C. Lysis released bacteria which have been already invasive before the addition of gentamycin and gave them the possibility to multiply on the agar plates. Anti-invasive activity was evaluated by counting CFU after incubation time. Bacteria incubated in control urine (day 1) served as untreated control.

#### *Motility assay*

Motility was evaluated using soft-agar plates (0.25 % agar), which were prepared the day prior to use and left at room temperature overnight. UPEC strain UTI89 (one colony from 24 h agar grown bacteria) were cultivated and suspended in 1 mL UPEC liquid medium. OD<sub>640 nm</sub> was adjusted to 20. Fifty µL of the suspension was mixed with 950 µL of the pooled urine samples and incubated for 2 h at 37 °C / 5 % CO<sub>2</sub>. One µL of the suspension was transferred into a soft agar plate, which was incubated for 48 h at 37 °C / 5 % CO<sub>2</sub>. Motility was determined by viewing wet mounts of bacterial cultures and measuring the diameter of motility.

## Quantitative real-time PCR (qPCR)

*Preparation of E. coli strain UTI89:* One colony of 24 h agar grown bacteria strain UTI89 was inoculated in 10 mL of pooled human urine (urine received independently of the study) and incubated overnight (~15 h) in a steady culture at 37 °C/5 % CO<sub>2</sub>. Bacteria were pelleted by centrifugation (5000 × g, 5 min), resuspended in 500 µL of fresh urine and the OD<sub>640 nm</sub> adjusted to 1. 100 µL of the suspension was transferred to 9.9 µL day 1 and day 8 urine samples and incubated at 37 °C/5 % CO<sub>2</sub> for 2 h to reach the mid-logarithmic phase. The suspensions were centrifuged (5000 × g, 5 min), the supernatant removed, and the pellets resuspended in 500 µL PBS. Immediately, 1 mL of RNAProtect Bacteria reagent was added to the pellet.

Bacterial RNA isolation: Total RNA was extracted using of RNeasy mini kit according to the manufacturer's instruction. Briefly, the tubes, which contained the bacteria and RNAProtect suspension were centrifuged (8.000 × g, 10 min), the supernatant was decanted, and 100 µL of TE buffer, containing 1 mg/mL lysozyme, were added to each tube. After 8 min of incubation at RT an appropriate volume of RLT buffer and ethanol were added. 700 µL of the mixture were transferred to an RNeasy spin column. After removal of impurities, 35 µL of RNase-free water were directly added to the column and the RNA was eluted by centrifugation.

*DNA digestion:* DNA was digested using a TURBO DNA-free Kit following the manufacturer's instruction. ≤200 µg/mL isolated RNA was transferred to a 0.2 mL tube and 5 µL of 10 × Turbo DNase buffer as well as 1 µL Turbo DNase was added mixed and incubated for 30 min at 37 °C in a Thermocycler. After adding 5 µL inactivation reagent and incubation for 5 min, the mixture is centrifuged (10.000 × g, 1.5 min) and the supernatant including RNA transferred to a fresh tube.

*cDNA synthesis:* RNA was transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit following the manufacturer's instruction. To 1 µg RNA (13 µL) 2 µL random hexamer primer was added. After 10 min incubation at 65 °C 4 µL reverse transcriptase buffer, 0.5 µL protector RNase inhibitor, 2 µL deoxynucleotide mix and 0.5 µL reverse transcriptase was added and the mixture incubated in the thermocycler (10 min 25 °C, 60 min 50 °C, 5 min 85 °C).

q-PCR was performed with an equivalent of 15 ng of total RNA using the iTaq Universal SYBR Green supermix (BioRad,) according to the protocol recommended by the manufacturer, using

a CFX96 Real-Time SystemC1000 Touch (BioRad). 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). Oligonucleotides were obtained from Eurofins MWG Operon, Luxembourg. Primer sequences used for the differential gene expression analysis are listed in Table 1.

#### *Tamm-Horsfall protein assay*

Concentration of THP in urine was quantified by an in-house sandwich-like ELISA, modified according [39]. 96-Well Nunc Maxisorp (Thermo Fisher) were coated with 100 µL of a solution (concentration 10 µg/mL) of wheat germ agglutinin from Triticum vulgaris (Sigma-Aldrich), diluted in coating buffer (pH 9.6, Na<sub>2</sub>CO<sub>3</sub> 50 mM, NaHCO<sub>3</sub> 349 mM, NaN<sub>3</sub> 0.02 % (w/v) in H<sub>2</sub>O) for 2 hours at room temperature while gently shaking. After rinsing with washing buffer (Tween 20, 0.05 % in PBS), non-specific binding sites were blocked with 200 µL of blocking buffer (2 % (m/v) BSA in washing buffer) for 2 h while gently shaking. After blocking, plates were washed 3 × with washing buffer. Residual buffer was removed by air drying.

Urine samples were diluted 1:10 (or 1:20 in cases where absorption at  $\lambda = 450$  nm was too high) with blocking buffer. 100 µL of the sample were added to the pre-coated wells and incubated for 2 h at room temperature while gently shaking. Samples and standards were run in duplicate. Blocking buffer served as blank. After incubation, plates were washed 3 × with washing buffer. Plates were then placed top-down on absorbent paper to remove residual buffer. Gentle tapping is recommended. 100 µL of Sheep Anti-Human Tamm-Horsfall Glycoprotein (BioRad) (1:1000 in blocking buffer) was dispensed in each well and incubated for 2 h at room temperature while gently shaking. Plates were washed as described above. 100 µL of Rabbit Anti-Sheep IgG (H+L)-HRP Conjugate (BioRad) (1:1000 in blocking buffer) were added and incubated for 1 h at room temperature. The well plate was washed again as previously described. For photometric detection, 100 µL of 3,3‘,5,5‘-tetramethylbenzidine (TMB Liquid Substrate System for ELISA) (Sigma-Aldrich) was dispensed on each well and incubated for up to 20 min in the absence of light. Measurements were performed immediately after addition of 100 µL sulfuric acid 4 mol/L at  $\lambda = 450$  nm with  $\lambda = 550$  nm as reference wavelength. The intensity of this signal is directly proportional to the concentration of THP present in the original

specimen. Concentration of THP was calculated by plotting a four-parameter logistic curve fit for standard concentrations and then interpolation of sample absorbances.

#### *Statistical analysis*

Statistical results were obtained using GraphPad Prism statistics (version 3) (GraphPad Software). Results are expressed as the mean value (MV)  $\pm$  standard deviation ( $\pm$  SD). Data ( $n \geq 3$ ) were processed by analysis of variance (one-way ANOVA). Subsequent post hoc test was conducted using Tukey's test to determine the statistical significance of differences between mean values of two with each other compared groups.  $p < 0.05$  was determined as statistically significant (\*),  $p < 0.01$  as high significant (\*\*) and  $p < 0.001$  as very high significant.

#### **Supporting Information**

Supplementary Data contain information on the UHPLC chromatogram of *O. stamineus* dry extract (Fig. 1S), standard parameters of test urines (Tables 1S and 2S), the osmolality of the urine samples (Table 3S).

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#### **Conflict of Interest**

The authors declare that they have no conflict of interest.

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**Table 1:** Primer sequences for qPCR of gene transcription in UPEC.

<b>Primer</b>	<b>Primer sequence 5'→3'</b>
<i>16S RNA</i>	forward: GCGGCATACAAAGAGAAG
<i>16S RNA</i>	reverse: ATGGAGTCGAGTTGCAGA
<i>fimH</i>	forward: CAATGGTACCGCAATCCCTA
<i>fimH</i>	reverse: GCAGGCGCAAGGTTACA
<i>fimC</i>	forward: CTAAATTAGCGTTGCCACCCG
<i>fimC</i>	reverse: AGGGTGTGGGTTAACAGC
<i>fimD</i>	forward: TCCGGTATGAATCTGCTGGC
<i>fimD</i>	reverse: CTGCTGACCCACATCCAGTT
<i>csgA</i>	forward: GTAGCAGCAATTGCAGCAATCG
<i>csgA</i>	reverse: TTAGATGCAGTCTGGTCAACAG
<i>papGIII</i>	forward: AGCAATTTCGGTTGGTCTG
<i>papGIII</i>	reverse: TCCACGCCATTAATCGAAAT
<i>papC</i>	forward: GATGGTGTGGGAGGGTGTACC
<i>papC</i>	reverse: CGCTTCAGGTCAACAGAGGT
<i>papD</i>	forward: CCCGGCAGCAATTAAAACCA
<i>papD</i>	reverse: ATACGATAACCCACCGCTGAC
<i>prsGIII</i>	forward: CAATTTCGGTTGGTCTGG
<i>prsGIII</i>	reverse: CGATGGTCAGGTTTG
<i>sfaG</i>	forward: AGCGGGTTCTGTGGTGAATA
<i>sfaG</i>	reverse: CCCGACATGAAATACCGACG
<i>focG</i>	forward: TGTTACAGGGAGGGTATTG
<i>focG</i>	reverse: GGTGCTGTTGGCTGCTAT
<i>fyuA</i>	forward: GGTCTTGATGCCAACCGTT
<i>fyuA</i>	reverse: GGTATAAAACGTCGCGGCTT
<i>chuT</i>	forward: GATTGCGGCTAACCTGAAG
<i>chuT</i>	reverse: TCAACGGTGATAATGCGCTG
<i>fliC</i>	forward: ACAGCCTCTCGCTGATCACTCAA
<i>fliC</i>	reverse: GCGCTGTTAATACGCAAGCCAGAA

## Legends to Figures

**Figure 1:** Influence of urine samples obtained after 7-day oral intake of *Orthosiphon* infusion on the bacterial adhesion of pre-incubated UPEC NU14 to human T24 bladder cells. **A:** Individual urine samples, all samples, no correction for outliers. **B:** Individual urine samples, corrected after Grubbs outlier test. **C:** Pooled urine samples from 20 volunteers. **D:** Urine sample from male and female individuals.

Data represent values from 3 independent adhesion assays, with  $n \geq 2$  technical replicates. PC: extract from *Zea mays* (1 mg/mL) [5]. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ .

**Figure 2:** Influence of pooled urine samples (Day 1, 3, 6, 8) on the proliferation of UPEC NU14 over a 24 h incubation period. PC: day 1 urine, supplemented with gentamycin (100 µg/mL). Data represent values from three independent experiments with  $n = 6$  technical replicates. Error bars represent mean  $\pm$  standard deviation.

**Figure 3:** Influence of pooled urine on the relative invasion [%], related to day 1 pooled control urine) of UPEC NU14 into T24 cells after 2 h pre-incubation (right) and comparison to the inhibition of bacterial adhesion to host cells (left). Data represent mean  $\pm$  standard deviation from 3 independent experiments with  $n \geq 2$  technical replicates. \*\*\*:  $p < 0.001$ .

**Figure 4:** Effect of pooled day 8 urine on the relative normalized gene expression of UPEC UTI89 after 2 h pre-incubation. Gene expression is normalized to the 16S rRNA is related to the untreated control urine group (day 1 urine = 1). Data represent mean  $\pm$  standard deviation from 3 independent experiments with  $n = 2$  technical replicates. \*\*\*:  $p < 0.001$ .

**Figure 5:** Effect of pooled urines from day 1 (= untreated control urine), day 3, 6, and 8 on the motility by swimming in soft agar. Cultures of 2 h pre-treated UPEC strain UTI89 were stabbed into 0.25 % soft agar plates and incubated at 37 °C for 48 h. **A:** Summary of results. Data represent mean  $\pm$  standard deviation from 3 independent experiments with  $n = 2$  technical replicates. **B:** Pictures of agar plates relative to the above tests (day 1: A, day 3: B, day 6: C, day 8: D).

**Figure 6:** Tamm-Horsfall Protein concentration in urine samples during a seven-day oral intake of Java tea as determined by ELISA. Data represent mean  $\pm$  standard deviation from  $\geq 2$  independent experiments and the respective median.

## **Legends to Tables**

**Table 1:** Primer sequences for qPCR of gene transcription in UPEC.