Supplementary Information

Traditional plant urologicals curb early uropathogenic *E. coli* infection and strengthen host innate defense

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Supplementary methods:

Minimum Inhibitory Concentration Assay

The MIC was determined by the microtiter broth dilution assay in Mueller-Hinton broth (MHB) as described before (Wiegand et al. 2008).

Growth curve assessment

To test the effect of plant extracts on bacterial growth, an overnight culture of *E. coli* CFT073-*gfp* was grown in M9 medium supplemented with casamino acids under shaking conditions at 37 °C. The next day, the overnight culture was diluted 1:200 in M9 medium supplemented with 0.4 % glucose and 0.4% casamino acids a microtiter plate. The plant extracts were added in concentrations similar to those used in the MIC assay (1 mg/ml as the highest final concentration), and absorption at λ = 595 nm was measured every 14 minutes at 37 °C in an Infinite F200 microplate reader (Tecan, Männedorf, Switzerland).

Counting of eukaryotic cells with the CASY Cell Counter & Analyzer

T24 cells were grown at 37 °C in 24-well plates at 1 × 10^5 cells per well and after a confluent monolayer was formed, they were incubated with plant extracts at different concentrations for 3 h 15 min. After that, the cells were trypsinized and counted using the CASY Cell Counter & Analyzer (OMNI Life Science, Bremen, Germany) according to the manufacturer's recommendations to assess the cell counts along with the cells' viability compared to the untreated control (Lindl et al. 2005).

Analysis of the viability of eukaryotic cells

T24 cells were cultured at 37 °C in a black 96-well plate with 1 × 10^5 cells seeded per ml (200 µl/well). After 48 h, when a confluent monolayer was formed, the cell culture medium was exchanged with fresh McCoy's medium, followed by the addition of plant extracts (final concentration was 330 µg/ml). They were incubated for 3 h 15 min, followed by the addition of 10% Alamar Blue reagent (v/v) to each well. The plate was then inserted into an Infinite F200 microplate reader (Tecan, Männedorf, Switzerland) for 24 h, measuring absorbance in 10 min cycles at λ = 595 nm and fluorescence with λ = 560 nm excitation and λ = 590 nm emission wavelengths.

References:

Lindl T, Lewandowski B, Schreyögg S, Stäudte A (2005) An evaluation of the *in vitro* cytotoxicities of 50 chemicals by using an electrical current exclusion method versus the neutral red uptake and MTT assays. Alternatives to Laboratory Animals 33:591–601. https://doi.org/10.1177/026119290503300614

Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175. https://doi.org/10.1038/nprot.2007.521

Supplementary Table 1: List of primers used in the study

Genes	Forward	Reverse
18S rRNA	5'-ATGGTTGCAAAGCTGAAACT-3'	5'-TCCCGTGTTGAGTCAAATTA-3'
GAPDH	5‘-CAAATTCCATGGCACCGTCA-3'	5‘-ATCTCGCTCCTGGAAGATGG-3‘
IL-6	5’-ACTCACCTCTTCAGAACGAATTG-3’	5’-CCATCTTTGGAAGGTTCAGGTTG-3’
IL-8	5’-ACTGAGAGTGATTGAGAGTGGAC-3’	5’-AACCCTCTGCACCCAGTTTTC-3’
CXCL-3	5’-AGCGTATCATTGACACTTCCTGC-3’	5’-TCCCTTTCCAGCTGTCCCTAG-3’
ß-Defensin	5’-CGCCATGAGAACTTCCTACC-3’	5’-TAAAGATCGGGCAGGCAGAA-3’
TLR4	5’-TGAGCAGTCGTGCTGGTATC-3’	5’-CAGGGCTTTTCTGAGTCGTC-3’
Prophenoloxidase	5'-TGTCCAATCCGCCGCAGTTTCCTA-3'	5'-CGCCAATAAGCAAGACGGTGTTCC-3'
Moricin	5'-GCGATCATTGCCCTCTTTAT-3'	5'-AGTGCCTTCTGTTTTTAATGTGTT C-3'
Hemolin	5'-CTCCCTCACGGAGGACAAAC-3'	5'-GCCACGCACATGTATTCACC-3'
Apolipophorin III	5'-AGACTTGCACGCCATCAAGA-3'	5'-TGCATGCTGTTTGTCACTGC-3'

Supplementary Table 2: Impact of plant extract A to D on the viability of T24 human bladder epithelial cells

Herbal extract	Concentration	Total count	Viability
A	330 µg/ml	3796	94.69 %
	990 µg/ml	3300	94.47 %
B	330 µg/ml	1733	88.92 %
	990 µg/ml	1696	90.31 %
C	330 µg/ml	3018	92.43 %
	990 µg/ml	1738	70.81 %
D	330 µg/ml	3228	93.46 %
	990 µg/ml	593	63.49 %
UC	-	3894	96.2 %

Supplementary Fig. 1: Determination of the minimum inhibitory concentration (MIC) of the plant extracts for *E. coli* CFT073-*gfp*. After 21 h of incubation with the plant extracts at concentrations ranging from 1 mg/ml to 0.03125 mg/ml, the absorption of the bacterial culture was measured at $\lambda = 595$ nm. The plant extracts showed no minimum inhibitory concentration for *E. coli* CFT073-*gfp* in the concentration range from 1 mg/ml to 0.03125 mg/ml.

Supplementary Fig. 2: Influence of plant extracts on the growth dynamics of *E. coli* CFT073-*gfp*. To examine the growth dynamics of *E. coli* strain CFT073-*gfp* in M9 medium in the presence of the tested plant extracts (concentration range from 1 mg/ml to 0.03125 mg/ml), absorption measurements were performed every 14 min for up to 700 min at λ = 595 nm. Compared to UC, there was no change in the growth rate.

Supplementary Fig. 3: Effect of plant extracts on biofilm formation of *E. coli* CFT073-*gfp*. *E. coli* CFT073-*gfp* was cultivated with 330 µg/ml plant extracts at 37 °C for 48 h, and biofilm formation was quantified by absorbance measurements at λ = 570 nm. The experiments were performed in biological replicates n = 3, each with n = 2 technical replicates, and compared with an untreated control. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 4: Regulation of the envelope stress response induced by plant extracts A and C in *E. coli* K-12 strain MG1655 (A-D for extract A and E-H for extract C). Fluorescence measurements to investigate the effect of the extracts on the Spy stress response using the wild-type strain MG1655 (A, E) and its *baeR*$^{-}$ (B, F) and *cpxR*$^{-}$ (C, G) mutants. The arrow indicates the time at which the extract was added to the bacterial culture. Comparison of total YFP expression between the WT strain and the regulatory mutants (D, H). The experiments were done with n=3 biological replicates and were compared with a water control. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 5: Effect of plant extracts on the viability of T24 bladder epithelial cells. The Alamar Blue assay shows that T24 cell viability was not impaired at a concentration of 330 µg/ml plant extracts. T24 cells were incubated with 330 µg/ml extracts, then 10% Alamar Blue reagent was added and the absorption and fluorescence were measured over 24 hours. The final endpoint fluorescence measurements for each of the plant extracts were normalized to the UC as a percentage of cell viability relative to the UC and plotted in the graphs (UC as 100% cell viability). For the negative control (NC), 10% DMSO was used. Data shown with biological replicates n=3, each with n=2 technical replicates and compared with untreated control. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 6: Effect of plant extracts on the invasion of *E. coli* CFT073-*gfp* into T24 cells. Concentration-dependent reduction in the number of intracellular bacteria when T24 cells were incubated with plant extracts at concentrations of 330 µg/ml, 120 µg/ml, and 75 µg/ml**.** Data shown with biological replicates n=3, each with n=2 technical replicates and compared with untreated control. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 7: Influence of plant extracts on UPEC invasion in human 5637 and RT-112 bladder epithelial cells. The number of intracellular UPEC in 5637 cells (A) and in RT-112 cells (B) is indicated. The experiments were done with n=3 biological replicates with n=2 technical replicates each and were compared with the untreated control, UC. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 8: Influence of plant extracts on the intracellular replication of *E. coli* CFT073-*gfp*. To assess the impact of the plant extract on early stages of UPEC infection (A), the T24 cells were incubated with extracts for 3 h and then infected with the UPEC strain for 24 h. The ratio of the number of infecting UPEC after 24 h compared to the 3 h value was calculated (in percent). To investigate the impact of the plant extracts on the later stages of UPEC infection, i.e., intracellular bacterial survival (B), the T24 cells were infected with UPEC for 2.5 hours and then treated with gentamicin for 1.5 h, before the plant extracts were added and allowed to stand for 24 h. Subsequently, the cells were lysed and plated. The experiments were done with n=3 biological replicates with n=2 technical replicates each and are compared with UC. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

Supplementary Fig. 9: Influence of plant extracts on the innate immune response in 5637 cells. Fold-change of IL-6, IL-8, CXCL-3, and β-defensin in 5637 cells and RT-112 cells (A,B for 5637 cells and C, D for RT-112 cells) after treatment with 330 µg/ml of extracts (A,C) IL-6, IL-8 expression and (B, D) CXCL-3, β-defensin expression. The fold-change was calculated relative to the untreated control and normalized to the 18S housekeeping gene in (A, B) and with GAPDH gene in (C, D). The experiments were done with n=3 biological replicates with n=2 technical replicates each and were compared with the untreated control, UC. *P* value < 0.05 is represented by *, ** is equivalent to a *p* value < 0.005, *** refers to *p* value < 0.0005, and **** refers to *p* value < 0.0001.

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