Influence of Cranberry Extract on Tamm-Horsfall Protein in Human Urine and its Antiadhesive Activity Against Uropathogenic *Escherichia coli*

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Bibliography
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ABSTRACT

LC-MS characterized cranberry extract from the fruits of Vaccinium macrocarpon inhibited under in vitro conditions the bacterial adhesion of *Escherichia coli* strain 2980 uropathogenic *E. coli* (UPEC strains UTI89, NU14) to T24 bladder cells and adhesion of UPEC strain CFT073 to A498 kidney cells in a concentration-dependent manner. Within a biomedical study, urine samples from 16 volunteers (8 male, 8 female) consuming cranberry extract for 7 d (900 mg/d) were analyzed for potential antiadhesive activity against UPEC by ex vivo experiments. Results indicated inhibition of adhesion of UPEC strain UTI89 to human T24 bladder cells. Subgroup analysis proved significant inhibition of bacterial adhesion in case of urine samples obtained from male volunteers while female urine did not influence the bacterial attachment. Differences between antiadhesive capacity of urine samples from male/female volunteers were significant. Protein analysis of the urine samples indicated increased amounts of Tamm-Horsfall protein (THP, syn. uromodulin) in the active samples. Inhibition of bacterial adhesion by the urine samples was correlated to the respective amount of THP. As it is known that THP, a highly mannosylated glycoprotein, strongly interacts with FimH of UPEC, this will lead to a decreased interaction with uroplakin, a FimH-binding transmembrane protein of urothelial lining cells. From these data it can be concluded that the antiadhesive effect of cranberry after oral intake is not only related to the direct inhibition of bacterial adhesins by extract compounds but is additionally due to an induction of antiadhesive THP in the kidney.

Introduction

Extracts and juices from cranberry fruits (*Vaccinium macrocarpon* Aiton, Ericaceae) have been investigated within a number of preclinical and clinical studies for prevention of UTI. A Cochrane meta-study concluded that cranberry products are not significantly different to standard antibiotic treatment for preventing UTI, but the evidence for a potentially significant benefit seems be too small for a clear recommendation for the prevention of UTI [1]. A strong heterogeneity of clinical data gets obvious when studying the available literature on the respective outcome from the clinical studies performed with cranberry extracts in humans. On one hand, this is due to the differently manufactured and thus chemically distinct extracts and juices used for medication. On the other side, a recent study indicated that also differences in the predominant adhesins expressed by UPEC, the major pathogens responsible for UTI, show different response toward cranberry extract. Only mannose-sensitive type 1 fimbriae bearing UPEC could be inhibited, while no interaction with P- and F1C-fimbriae dominated UPEC was observed [2].

Concerning the potential mode of action, cranberry extracts are claimed to inhibit UPEC attachment to bladder epithelial cells [3]. Additionally, inhibition of bacterial flagella expression and motility has been described [4, 5]. Also, effects of cranberry ex-

ABBREVIATIONS

CDE-Q	cranberry dry extract Nutrican
CFU	colony forming units
FITC	fluorescein isothiocyanate
GCP	good clinical practice
GMP	good manufacturing practice
HRP	horseradish peroxidase
PAC	proanthocyanidin
UPEC	uropathogenic *Escherichia coli*
THP	Tamm-Horsfall protein
UTI	uncomplicated urinary tract infections

tracts on bacterial biofilm formation have been discussed with non-convincing and contradictory results [5, 6]. Tapiainen et al. [7] observed even increased biofilm formation after *in vivo* intake of cranberry juice for 20 % of the UPEC strains.

In older literature, the inhibition of bacterial adhesion was described to be due to the presence of A-type PAC trimers, which were claimed to interact with P-receptor-coated beads with immobilized [α-D-Gal-(1–4)-β-D-Gal]-disaccharide [3, 8]. This theory has been rebutted recently, as also PAC-free extracts have significant antiadhesive effects within *in vivo* studies in humans, which are due to an interaction with type 1 fimbriae of UPEC but not with P and F1C-fimbriae dominated bacteria [2].

It remains unclear what secondary natural product from cranberry extract is responsible for this inhibition of the bacterial adhesion. During examination of the complex literature published on cranberry activity against UPEC, it seems astonishing that most of the authors claim PACs to be responsible for the antiadhesive activity. On the other hand, recent studies have shown that PACs get absorbed over the intestinal barrier only to a very limited extent and these oligomers are not bioavailable in relevant concentrations [9, 10]. Interestingly, polyphenols known to be microbial-derived metabolites of PACs from intestinal degradation–for example, phenylacetic acid, 3,4-dihydroxyphenylacetic acid and catechol–have recently been identified as antiadhesive compounds against UPEC [11], and also myricetin has been pinpointed as an antiadhesive compound in human urine from volunteers after cranberry uptake [12]. From the current state of knowledge, it is assumed that PACs are not predominantly responsible for the antiadhesive effects of cranberry, while PAC metabolites, formed during the intestinal passage or cranberry-associated flavonoids, are bioavailable to a significant extent and can be found as potential antiadhesive compounds in the urine [13].

The purpose of the following investigations was to initiate an *in vivo* biomedical study in healthy volunteers for systematic evaluation of potential antiadhesive activity against UPEC in the urine after oral treatment with a fully characterized cranberry extract. Additionally, the influence of the urine on UPEC adhesion to bladder cells within *ex vivo* studies was to be studied, and the respective bioactivity was to be correlated to the respective metabolome. Surprisingly, it became obvious during the evaluation of the *ex vivo* assay that inhibition of the bacterial adhesion by cranberry extract seems to be strongly gender-specific. This report documents for the first time the situation that urine from cranberry-treated men significantly reduces under *ex vivo* condition the bacterial attachment of UPEC to bladder cells, which was not the case when urine from female volunteers had been used.

Results

For quality control, food-grade cranberry dry extract CDE-Q was characterized using LC-ESI-DAD-qTOF-HR-MS (▶ **Fig. 1**). Peaks were assigned to the respective secondary compounds as displayed in Table 1, based on comparison of pseudomolecular ions and major fragments' exact *m/z* values with published data of *V. macrocarpon* constituents. CDE-Q is specified by the manufacturer as spray-dried cranberry concentrate powder standardized to 2.7 % PACs. CDE-Q was investigated on potential cytotoxic effects against UPEC strain UTI89 by determination of bacterial growth in liquid culture over 24 h. Even with CDE-Q concentrations as high as 2500 µg/mL, no inhibition of bacterial growth was observed (data not shown). CDE-Q concentrations of up to 1000 µg/mL did not influence the viability of eukaryotic T24 bladder cells over 24 h incubation time as analyzed by MTT assay (data not shown).

Co-incubation of T24 cells together with *Escherichia coli* strains 2980 or UTI89 under *in vitro* conditions together with CDE-Q (100–2500 µg/mL) and evaluation of the bacterial adhesion to the host cells by flow cytometry resulted in a concentration-dependent decrease in bacterial adhesion (▶ **Fig. 2 A** and **B**). Similar results were obtained by monitoring the bacterial adhesion of UPEC strain CFT073 on human A498 kidney cells (▶ **Fig. 2 C**), which was also significantly reduced in the presence of CDE-Q.

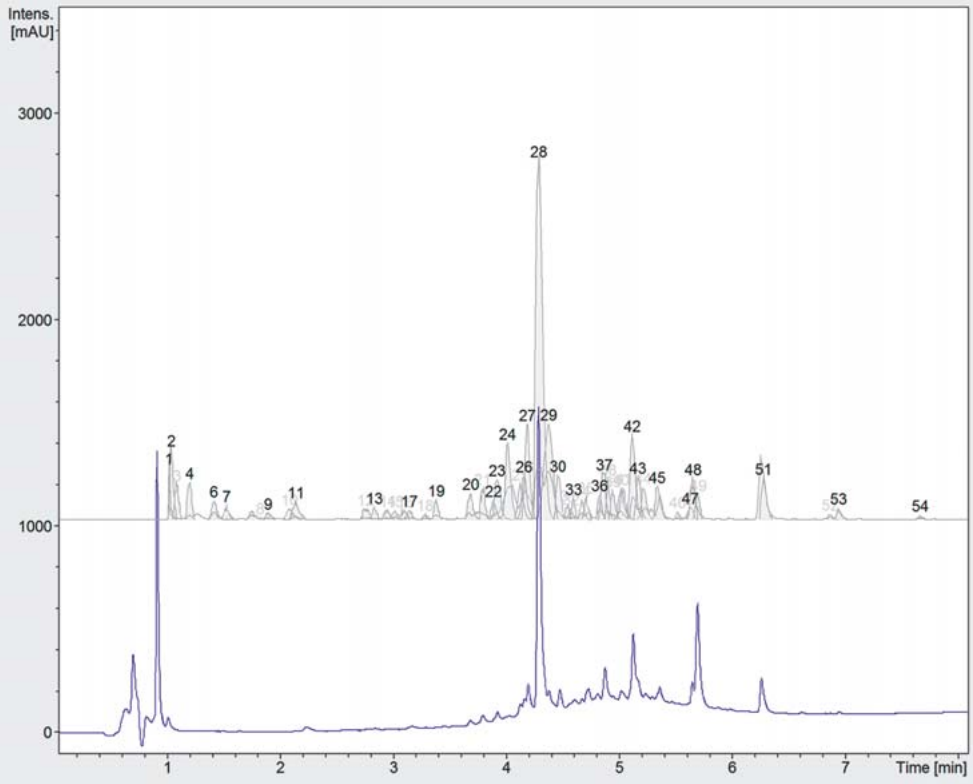
In addition, pre-incubation of UPEC strain NU14 with CDE-Q also resulted in significant and concentration-dependent effects (data not shown).

These *in vitro* findings correlate qualitatively and quantitatively with previously reported data on inhibition of bacterial adhesion to T24 bladder cells by cranberry extract [2]. The slightly higher inhibition rates of CDE-Q against *E. coli* UTI89 might be due to the fact that the main bacterial adhesin of UTI89 is FimH, which displays a higher mannose affinity in this strain than the FimH variant of UPEC strain CFT073 [14].

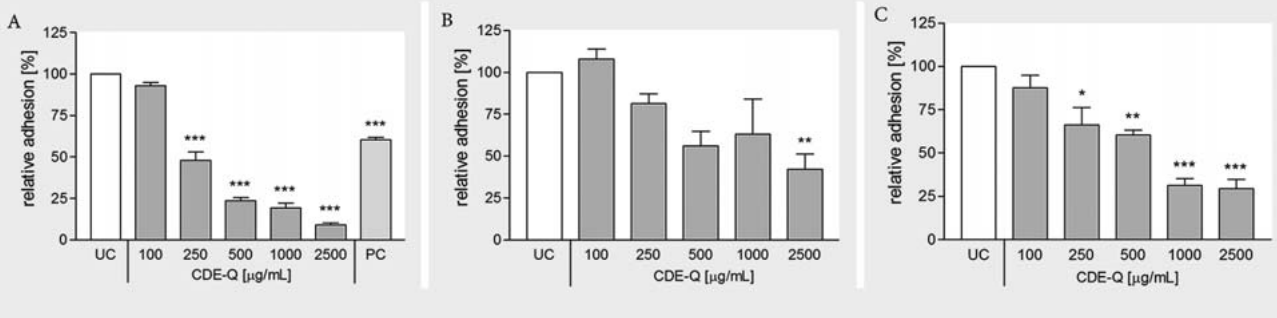
Within the scope of the 7-day treatment with cranberry extract in the context of the biomedical *ex vivo* study, no intolerances or drop-outs were observed. The monitoring of morning urine samples obtained from the volunteers before (day 0) and after treatment (days 2, 4, 6, 8) with CDE-Q showed no significant differences for creatinin, pH, leukocytes, erythrocytes, sodium, potassium, chloride, bilirubin, glucose, and ketones between the treated and nontreated samples.

The urine samples were tested *ex vivo* using 3 different *E. coli* strains (UTI89, NU14, and 2980) using 2 different flow-cytometry-based assay protocols: co-incubation of fluorescent-labeled bacteria, together with T24 bladder cells, and urine samples and pre-incubation of the bacteria for 48 h with the urine samples.

Both the individual urine samples from the volunteers and the pooled urine from 8 women and 8 men obtained during the different treatment intervals were tested separately in the pre- and co-incubation protocols.



▶ **Fig. 1** Full-MS chromatogram (gray) and UV chromatogram (violet, λ = 230 nm) of cranerry dry extract CDE-Q. For peak assignment by MS, see ▶ **Table 1**.



▶ **Fig. 2** Relative adhesion of *E. coli* strains UTI89 (**A**) and 2980 (**B**) to T24 bladder cells and CFT073 to A498 kidney cells (**C**) after co-incubation of bacteria, cells, and cranberry extract CDE-Q under *in vitro* conditions. Data are related to the bacterial adhesion of untreated control groups (UC = 100 %). PC: positive control, mannose (2.8 mM). *** $p < 0.001$, values represent mean ± SD from 3 independent experiments with n = 2 technical replicates.

As displayed in ▶ **Fig. 3**, bacterial adhesion of *E. coli* strains UTI89 and 2980 to the host cells was reduced by the pooled urine samples over the time, with a (nonsignificant) inhibition rate of 37.7 ± 16.9 % (UTI89) and 35.3 ± 12.5 % (2980) at day 8 (▶ **Fig. 3 A** and **B**). Testing of the individual urines in the co-incubation protocol for potential antiadhesive activity against UPEC revealed in total an inconsistent data set with a time-dependent tendency for inhibition of bacterial adhesion at day 8 (▶ **Fig. 4**).

No effect of CDE-Q on bacterial invasion into the host was observed (data not shown).

Interestingly, subgroup analysis of the individual samples by differentiation into test samples obtained from male and female volunteers indicated time-dependent significant antiadhesive activity of urine samples obtained from men (▶ **Fig. 5 A** and **B**). In contrast, urine samples from women did not show any significant influence on the bacterial adhesion (▶ **Fig. 5 C** and **D**).

▶ **Table 1** LC-qTOF-MS peak characteristics as obtained by integrating a full-MS chromatogram (gray) of cranberry extract CDE-Q. Peaks were assigned to known constituents of *V. macrocarpon* as denominated in the references [35–39]. Assignment of stereoisomers is based on published RP18 elution order and relative peak areas. Assignments without references are based on the *de novo* interpretation of MS, MS^2, and UV-spectra. Peaks 25, 30, 32, 43, 44, 47, 49, 50, and 51 are constituted of 2 compounds, respectively. Chemical structures are displayed in the cited literature. n. a.: not accessible.

Peak	tR/min	*m/z* (Ion)	Ion	Fragment *m/z*	Λ_{max} (nm)	Area fraction	Compound	Reference	Molecular formula	Error	mSigma
1	1.021	413.1093	M + Na		236	1.2 %	not identified				
		275.1130	base peak				not identified				
2	1.042	132.1028	base peak		236	2.8 %	not identified				
3	1.091	171.0281	base peak		203, 230, 275	1.4 %	not identified				
4	1.204	293.1256	M + H		200, 243, 274	1.6 %	not identified				
5	1.267	207.0538	[M + H]		231, 271	0.8 %	not identified				
6	1.416	243.0868	M + Na		212, 284	1.0 %	not identified				
7	1.526	166.0878	M + H	120.0815	230, 292	0.6 %	phenylalanine	mzCloud	$C_9H_{11}NO$	− 1.5 mDa	5.7
8	1.826	461.2164	base peak		240, 284	0.5 %	not identified				
9	1.896	323.1120	M + Na	205.0876	244, 276	0.4 %	not identified				
10	2.085	163.0606	M + H		276	0.7 %	not identified		$C_6H_{10}O_5$	− 1.5 mDa	5.9
11	2.141	317.1256	M + H		260	1.1 %	glycoside of $C_6H_{12}O_3$		$C_6H_{12}O_3$	− 2.3 mDa	48.3
12	2.754	595.1509	M + H	303.0539	284, 305 sh	0.7 %	prodelphinidin B3		$C_{30}H_{26}O_{13}$	6.3 mDa	12.7
13	2.835	349.0915	M + Na	165.0563	292	0.5 %	coumaroyl-hexose	[39]	$C_{15}H_{18}O_8$	− 2.1 mDa	28.5
14	2.950	205.0984	M + H	188.0721	280, 310	0.5 %	tryptophane	mzCloud	$C_{11}H_{12}N_2O_2$	1.3 mDa	22.7
15	3.017	265.1573	base peak	265.1573	204, 280, 368	0.5 %	not identified				
16	3.111	354.1198	M + H	192.068, 163.0474	204, 280, 320	0.5 %	3-caffeoylquinic amide		$C_{16}H_{19}NO_8$	1.4 mDa	14.3
17	3.144	1153.2722	M + H	577.1381	280	0.5 %	A-type procyanidin tetramer	[38]	$C_{60}H_{48}O_{24}$	11.3 mDa	145.3
18	3.283	579.1552	M + H	289.0698	204, 280	0.2 %	procyanidin B2	[40]	$C_{30}H_{26}O_{12}$	− 9.6 mDa	26.4
19	3.383	383.1332	M + Na	181.0862		0.8 %	caffeoyl derivate				
20	3.686	343.1029	M + H	181.0522	204, 284, 305 sh	1.3 %	caffeoyl glucose	[39]	$C_{15}H_{18}O_9$	− 0.5 mDa	72.5
21	3.792	355.1047	M + H	163.039	204, 296, 324	1.3 %	chlorogenic acid	mzCloud	$C_{16}H_{18}O_9$	2.3 mDa	16.9
22	3.887	162.0548	M + H		204, 284	0.9 %	not identified				
23	3.919	349.0912	M + Na	147.0443	204, 284	2.4 %	coumaroyl hexose		$C_{15}H_{18}O_8$	− 1.8 mDa	8.2
24	4.011	419.1013	M + H	287.1101	204, 280	4.1 %	kaempferol pentose		$C_{20}H_{19}O_{10}$	− 4.0 mDa	7.7
25	4.125	463.1277	M +	301.0738+	280, 313 sh	1.9 %	peonidin-3-galactoside	[41]	$C_{22}H_{23}O_{11}$+	− 4.3 mDa	38.5
		579.1552	M + H	409.0968, 287.0583			B-type procyanidin dimer	[38]	$C_{30}H_{26}O_{12}$	− 5.5 mDa	9.5
26	4.158	463.1281	M +	301.0738+	282, 320 sh	2.3 %	peonidin-3-galactoside	[35, 41]	$C_{22}H_{23}O_{11}$+	− 4.6 mDa	6
27	4.184	463.1281	M +	301.0738	282, 320 sh	4.8 %	peonidin-3-galactoside	[35]	$C_{22}H_{23}O_{11}$+	− 4.4 mDa	12.8

cont.

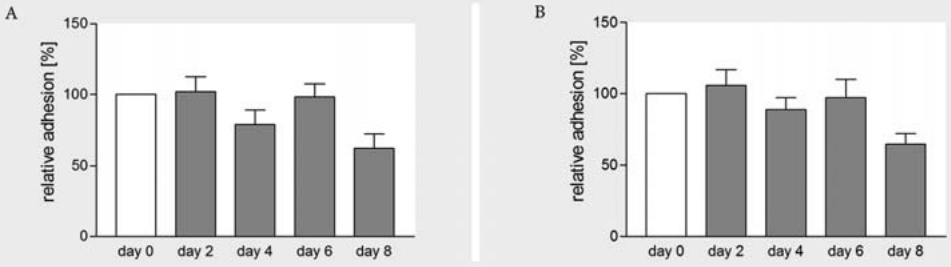
▶ **Table 1** *Continued*

Peak	tR/min	*m/z* (Ion)	Ion	Fragment *m/z*	Λ_{max} (nm)	Area fraction	Compound	Reference	Molecular formula	Error	mSigma
28	4.285	307.0818	M + Na	285.0992, 163.0601, 123.0445	232, 280	24.2 %	benzoyl hexose		$C_{13}H_{16}O_7$	− 3.0 mDa	4.3
29	4.373	433.1166	M + H	301.0733	232, 280	8.5 %	peonidin-3-arabinoside	[35, 41]	$C_{21}H_{21}O_{10}^+$	3.6 mDa	3.2
30	4.458	387.2015	M + H		204, 280	2.2 %	not identified				
		865.2055	M + H	695.1475, 577.1469, 451.1122, 411.1116, 289.0730			A-type procyanidin trimer	[38]	$C_{35}H_{36}O_{18}$	8.0 mDa	130.4
31	4.548	1153.2742	M + H	577.1454	204, 280	0.5 %	A-type procyanidin tetramer	[38]	$C_{60}H_{48}O_{24}$	− 13.4 mDa	57.4
32	4.572	1155.2838	M + H	865.2051	204, 280	0.4 %	B-type procyanidin tetramer	[38]	$C_{60}H_{50}O_{24}$	− 7.3 mDa	206.7
		1153.2721	M + H				A-type procyanidin tetramer		$C_{60}H_{48}O_{24}$	− 11.3 mDa	332.2
33	4.598	207.1389	base peak		204, 280	0.7 %	not identified				
34	4.67	481.1013	M + H	319.0478, 153.0171	204, 276, 348	0.8 %	myricetin-hexoside	mzCloud	$C_{21}H_{20}O_{13}$	3.7 mDa	16.6
35	4.707	865.2040	M + H	577.1438	204, 276	0.8 %	A-type procyanidin trimer	[38]	$C_{45}H_{36}O_{18}$	6.5 mDa	6.4
36	4.828	321.1100	M + H	146.0602	204, 288	0.9 %	not identified				
37	4.868	577.2060	M + H	415.1518, 188.0711, 165.0557	204, 292	2.3 %	coumaroyl tryptophan derivate		$C_{27}H_{32}N_2O_{12}$	3.2 mDa	29.4
38	4.9	343.0922	base peak		204, 308	1.2 %	not identified				
39	4.941	579.2186	M + H	417.1667	204, 281, 305 sh	1.3 %	coumaroyl tryptophan derivate		$C_{27}H_{34}N_2O_{12}$	− 0.2 mDa	10.1
40	5.014	465.1042	M + H	303.0512, 153.0151	204, 280	1.8 %	quercetin-hexoside	[39]	$C_{21}H_{20}O_{12}$	− 1.5 mDa	18.6
41	5.04	519.1534	base peak		204, 280	1.7 %	not identified				
42	5.114	577.1384	M + H	425.0883, 287.0575	204, 280	4.3 %	B-type procyanidin dimer	[39]	$C_{30}H_{26}O_{12}$	− 4.3 mDa	5.3
43	5.163	435.0931	M + H	303.0498	204, 280	2.0 %	quercetin-pentoside		$C_{20}H_{18}O_{11}$	− 0.9 mDa	17.4
		577.1378	M + H	287.0578			A-type procyanidin dimer	[38]	$C_{30}H_{24}O_{12}$	3.7 mDa	23.2
		865.2026	M + H				A-type procyanidin trimer		$C_{45}H_{36}O_{18}$	5.2 mDa	51.8
44	5.21	435.0953	M + H	303.498	204, 272	1.6 %	quercetin-pentoside		$C_{20}H_{18}O_{11}$	3.1 mDa	85.3
		449.1800	M + Na	265.1424			not identified				
45	5.332	347.0771	M + H	331.0357, 303.0719	204, 225 sh, 266, 348	1.7 %	dimethylmyricetin (syringetin)	mzCloud, [39]	$C_{17}H_{14}O_8$	− 4.4 mDa	4
46	5.512	209.1150	base peak		204, 225 sh, 280	0.3 %	not identified				
47	5.627	449.1118	M + H	317.0672	220 sh, 266 sh, 372	0.7 %	methoxyquercetin-pentoside	[42]	$C_{21}H_{20}O_{11}$	− 4.0 mDa	31.9
		319.0466	M + H	217.0504, 153.0186			myricetin	mzCloud	$C_{15}H_{10}O_8$	1.7 mDa	87.9

cont.

▶ **Table 1** *Continued*

Peak	tR/min	*m/z* (Ion)	Ion	Fragment *m/z*	Λ_{max} (nm)	Area fraction	Compound	Reference	Molecular formula	Error	mSigma
48	5.648	319.0469	M + H	217.0504, 153.0186	220, 267 sh, 372	2.1 %	myricetin	mzCloud	$C_{15}H_{10}O_8$	− 2.0 mDa	10.5
49	5.694	319.0470	M + H	153.0184	200, 228, 265, 372	1.1 %	myricetin	mzCloud	$C_{15}H_{10}O_8$	2.1 mDa	75.4
		123.0442	M + H				benzoic acid		$C_7H_6O_2$	− 0.2 mDa	9.5
50	5.718	479.1190	M + H	317.0462	200, 228, 272	0.4 %	isorhamnetin-pentoside	[39]	$C_{22}H_{22}O_{12}$	− 0.6 mDa	24.2
		347.0775	M + H				dimethylmyricetin	[39]	$C_{17}H_{14}O_8$	− 1.3 mDa	15.9
51	6.273	569.1326	M + H	303.0521, 153.0178	208, 252, 368	2.1 %	3-O-(6′′-O-benzoylglucosyl) quercetin (neobignonoside)	[39]	$C_{28}H_{24}O_{13}$	− 3.7 mDa	12.7
		333.0619	M + H	153.0229			methylmyricetin		$C_{16}H_{12}O_8$	1.4 mDa	7.2
52	6.861	347.0765	M + H		220, 272, 368	0.3 %	dimethylmyricetin		$C_{17}H_{14}O_8$	− 0.3 mDa	12.8
53	6.936	317.0669	M + H		224, 272, 368	0.5 %	isorhamnetin		$C_{16}H_{12}O_7$	1.3 mDa	5.8
54	7.658	195.1177	M + H + H_2O		224, 280	0.2 %	not identified				



▶ **Fig. 3** Relative adhesion of *E. coli* strains UTI89 (**A**) and 2980 (**B**) to T24 bladder cells during co-incubation for 1 h (UTI89) or 2 h (2980) with pooled urine samples obtained from cranberry extract CDE-Q-treated volunteers (n = 5 male + 5 female, 900 mg extract for 7 d). Day 0: control urine, prior to CDE-Q consumption; days 2, 4, 6, 8: urine obtained at different days during CDE-Q application. Data are related to the initial adhesion values, determined for day 0 (= 100 %). Values represent mean ± SD from 3 independent experiments with n = 2 technical replicates.

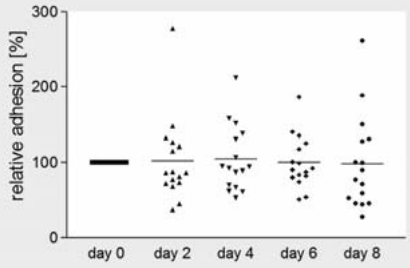
▶ **Fig. 6** displays the changes in the antiadhesive capacity of the individual urine samples obtained from men and women over the time. It is obvious from this evaluation that 7 from 8 test samples from men showed time-dependent effects on the bacterial adhesion. In contrast, incubation in female urine led even to increased bacterial adhesion with a much higher variance.

Pre-incubation of the bacteria for 48 h with the urine test samples and evaluation of the antiadhesive capacity against UPEC strain UTI89 again indicated a tendency but no significant inhibition of the bacterial adhesion (▶ **Fig. 7 A** and **B**). Subgroup analysis did not indicate significant differences between urine samples from men and women, due to the high variance. Improved data were obtained when analyzing the pooled urine against strains UTI89 and NU14 (▶ **Fig. 7 C** and **D**), indicating significant antiadhesive effects.

Intensive LC-MS analysis of the urine samples for identification of cranberry-related metabolites in combination with multivariate statistics did not lead to the identification of distinct compounds (data not shown). *In vitro* testing on antiadhesive activity against UPEC of a very high number of natural products related to cranberry extracts and also respective human metabolites from cranberry polyphenols did not indicate any superior inhibitory activity of individual compounds. At this point, the hypothesis was created that the antiadhesive effect observed by the urine samples could be due to an endogenous compound released from the human organism itself. As it is known that one of the most promi-

nent proteins in human urine is THP (syn. uromodulin) [15] and THP as a part of the innate immune system can strongly modulate endogenous defense strategies, all urine samples obtained from the biomedical study were investigated by ELISA on the amount of THP. Data displayed in ▶ **Fig. 8A** indicated that the pooled urine samples from 10 volunteers (5 women, 5 men) treated over 7 d with cranberry extract CDE-Q showed THP concentrations that increased very slightly over the time. Significance against the day 0 values was not detectable. Pooled urine from female volunteers (▶ **Fig. 8A**) showed nonsignificant decrease of THP titers. THP titers in urine samples obtained from male volunteers increased significantly from day 0 to day 8 (▶ **Fig. 8A**).

THP quantification in the individual urine samples of 16 volunteers (▶ **Fig. 8B**) indicated an increase in THP concentrations from day 0 to day 8 in the group of male volunteers within 5 out of 8 urine samples; 3 out of 8 showed decrease titers. In contrast, 6 samples out of 8 from the female urine samples showed decreased THP titers, while only 2 of 8 had increased THP concentrations.



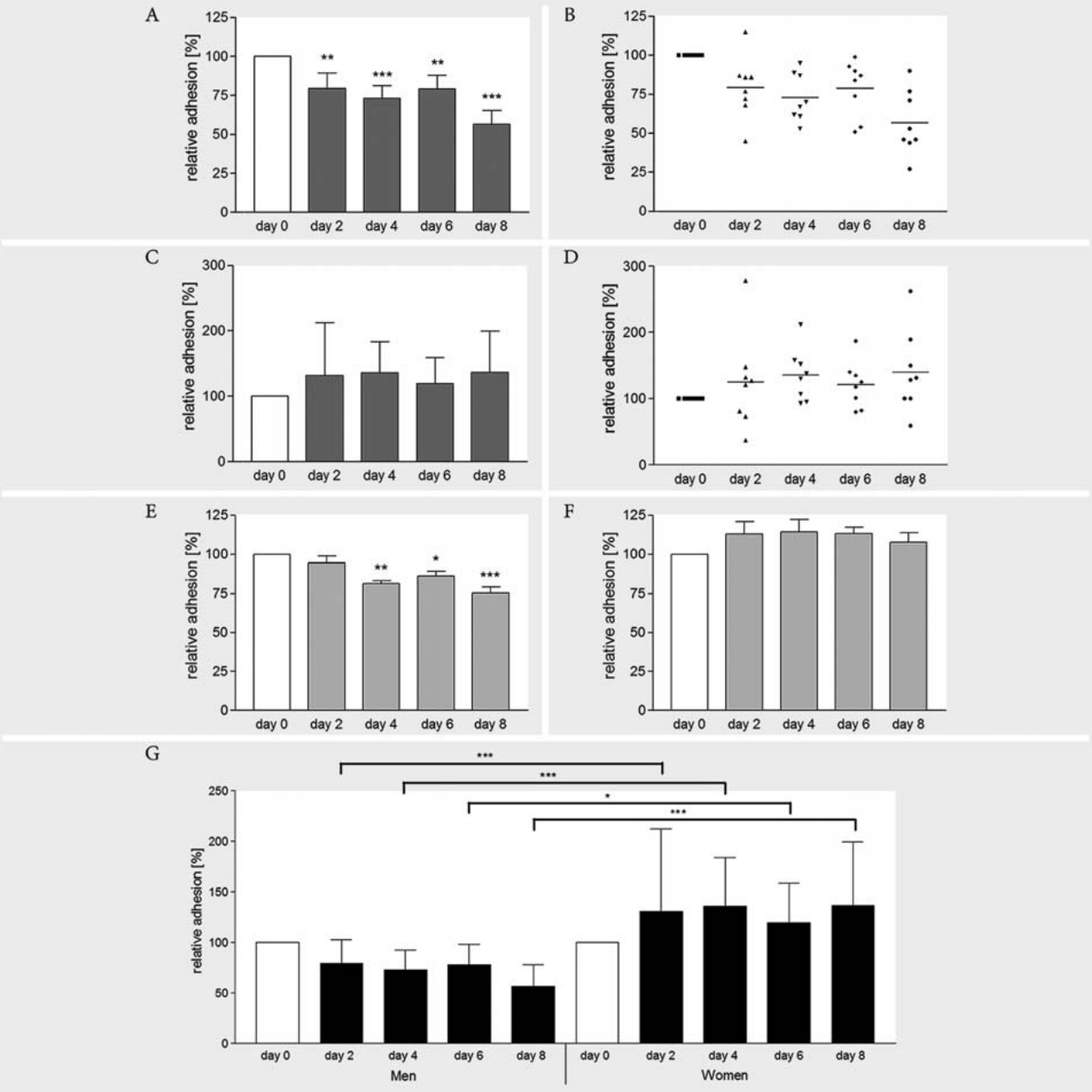
▶ **Fig. 4** Relative adhesion of *E. coli* strains UTI89 to T24 bladder cells during co-incubation for 1 h with individual urine samples obtained from cranberry extract CDE-Q-treated volunteers (n = 8 male + 8 female, 900 mg extract for 7 d). Day 0: control urine, prior to CDE-Q consumption; days 2, 4, 6, 8: urine obtained at different days during CDE-Q application. Data are related to the initial adhesion values, determined for day 0 (= 100 %). Bars represent the respective mean value of the respective group from 3 independent experiments.

Discussion

The clinical efficacy of cranberry extracts for prevention of UTI has been reviewed by a systematic Cochrane meta-analysis [1], indicating a small trend toward fewer UTIs in people taking cranberry products compared to placebo or no treatment. This was, however, not a significant finding [1]. This nonsignificance is discussed by the authors by the fact that the products used in the studies had been quite diverse concerning their composition. Juices have been used, as well as a variety of nonstandardized extracts. On the other hand, it is known from combined *in vivo*/*ex vivo* studies that antiadhesive activity of cranberry extracts depends also on the predominant adhesin types expressed by individual UPEC strains [2]. The bacterial adhesion of FimH dominated UPEC can be inhibited by cranberry extracts, while PapG-dominated strains are not susceptible [2]. Additionally, cranberry extracts are highly complex mixtures from various classes of natural products, including carbohydrates, polysaccharides, organic acids, flavonoids, oligomeric PACs, terpens, and many others. As shown in previous studies from different groups PAC metabolites [11, 16], anthocyanidins [17], flavonoids [18], and short-chain organic acids [19] might influence under *in vitro* conditions the bacterial adhesion. These *in vitro* data in most cases do not reflect the real pharmacokinetic properties of these compounds and are in part contradictory in the relevant literature ([11] vs. [16]). This uncertainty and high variability explains from our point of view why the clinical outcome until now is not as clear as it could be. Therefore, it seems essential to develop a standardized cranberry drug formulation, with known composition, standardized content, GMP-conform manufacture, documented stability, and clinical investigation according to the international GCP guidelines. Use of nonstandardized functional food or food supplement products might not be the right way toward a rationalized use of cranberry products against UTI.

Despite these problems, antiadhesive plant-derived formulations can have an increasing impact on prevention and therapy of UTI. The transfer of antiadhesive effects observed with plants extracts under *in vitro* conditions has been rationalized also by mouse infection models after oral application of extracts from *Orthosiphon stamineus* leaves [20] and *Apium graveolens* [21]. In both cases, significantly reduced infection rates in bladder and kidney tissues were observed, indicating that *in vitro* data from antiadhesive extracts and isolated compounds do correlate to *in vivo* reality after oral ingestion.

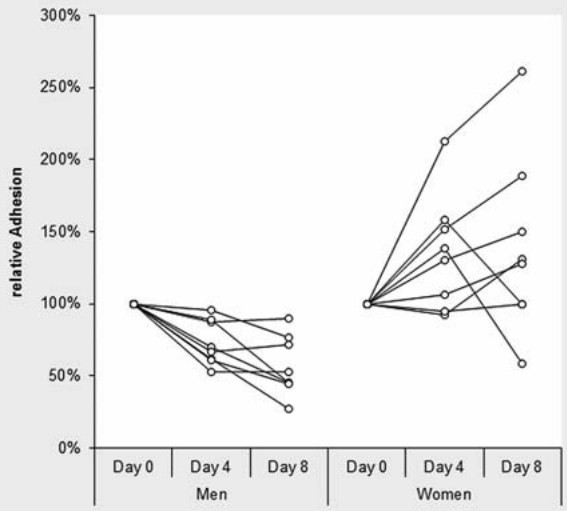
Within the present study, a fully characterized cranberry extract, for which antiadhesive potential under *in vitro* conditions against different UPEC strains has been shown, was administered to humans and the antiadhesive capacity of urine samples during the intervention was monitored by *ex vivo* adhesion assays against UPEC. The initial outcome of this biomedical study was comparable to results reported by previous studies [2]: the bacterial adhesion was decreased, but standard deviation was large and only limited significance was reached–similar to the observations from other studies. Interestingly, subgroup analysis clearly indicated gender-specific effects: urine samples from male volunteers had significantly higher antiadhesive capacity compared to samples obtained from women. From our point of view, this gender-specific action has not been reported for cranberry treatment until now. The reason for this can be seen in the higher concentrations of THP in the urine samples obtained from male volunteers; a significant and time-dependent increase in THP was obvious after cranberry intake. As THP is known to be part of the innate immune defense and is produced exclusively by the renal tubular cells in the Henle loop of the kidney, we assume that cranberry metabolites (which are not known until now) stimulate the expression and secretion of THP into the urine. The glycoprotein THP is characterized by conserved high-mannose moieties, which specifically binds to type 1 fimbriated UPEC. As type 1 fimbriae are known to interact with mannose residues from uroplakin on the surface of uroepithelial cells, which serves as adhesion receptor for the bacterium, THP abolishes the binding of the bacteria to uroplakins [22].



▶ **Fig. 5** Relative adhesion of UPEC strain UTI89 to T24 bladder cells after co-incubation with urine obtained from cranberry extract CDE-Q-treated volunteers (n = 16, 900 mg extract for 7 d). **A** Mean adhesion values obtained from individual urine test samples from 8 male volunteers, UPEC UTI89; **B** individual urine test samples from 8 male volunteers, UPEC UTI89; **C** mean adhesion values obtained from individual urine test samples from 8 female volunteers, UPEC UTI89; **D** individual values obtained from urine test samples from 8 female volunteers, UPEC UTI89; **E** antiadhesive activity of pooled urine from 8 male volunteers, UPEC UTI89; **F** antiadhesive activity of pooled urine from 8 female volunteers against UPEC UTI89; **G** comparative ANOVA evaluation of relative bacterial adhesion to T24 bladder cells determined for UTI89 during co-incubation treatment with urine samples from men and women. Data are related to the initial adhesion values determined for day (= 100 %). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$. Values represent mean ± SD from 3 independent experiments.

In principle, this means that the observed antiadhesive effect of cranberry extracts is not exclusively due to a direct interaction of metabolites with the bacteria but also to the stimulation of kidney cells to secrete higher amounts of THP, which prevents binding of UPEC to host cells via uroplakins.

This mechanism could also explain the situation that many studies, which have been performed to identify antiadhesive compounds from cranberry, failed more or less, as in all cases bladder cells have been used for adhesion assays. As THP is only produced



▶ **Fig. 6** Relative adhesion of UPEC strain UTI89 to T24 bladder cells after co-incubation with urine obtained from cranberry extract CDE-Q-treated male and female volunteers (n = 16, 900 mg extract for 7 d) at day 4 and day 8. Each dot represents 1 volunteer from 3 independent experiments.

in kidney cells, not in bladder cells, this indirect antiadhesive mechanism will not be observed in these *in vitro* assays.

For a future pinpointing of antiadhesive compounds from cranberry extract, the scientific question to be answered should be not "which compounds in the extract have antiadhesive activity against UPEC?" but "which compounds from cranberry stimulate the kidney to secrete increased amounts of THP?" In addition, the observed gender-specific effect on THP secretion caused by cranberry extract warrants further investigation. A recent study has revealed no differences in THP levels between men and women [23]. Use of primary cells of defined donors for *ex vivo* investigations should clarify this phenomenon. We assume that the response of the different biological subjects to cranberry metabolites in regards to THP secretion in the ascending limb of the loop of Henle might be different between men and women, but this has to be clarified in detail by follow-up studies. Additionally, it can be discussed that the degree of mannosylation of the secreted THP might be different between men and women; the higher the glycosylation, the higher should be the anti-FimH activity of the THP against UPEC.

We assess at this point the story around cranberry for UTI as follows: the multicomponent preparation cranberry extract exerts a dual activity against the adhesion of UPEC. On one side, a direct inhibition of FimH-mediated adhesion is observed, probably due to polyphenolic metabolites. Additionally, cranberry stimulates secretion of THP in the kidney, which again acts as a strong inhibitor of type 1 fimbriae adhesion. Both aspects together prevent bacterial adhesion and can act positively for prevention of UTI.

Materials and Methods

Materials

If not stated otherwise, solvents and reagents were obtained from VWR International; consumables were obtained from Sarstedt. All solvents and reagents were of analytical quality. Water was produced by a Millipore Simplicity 185 system (Merck).

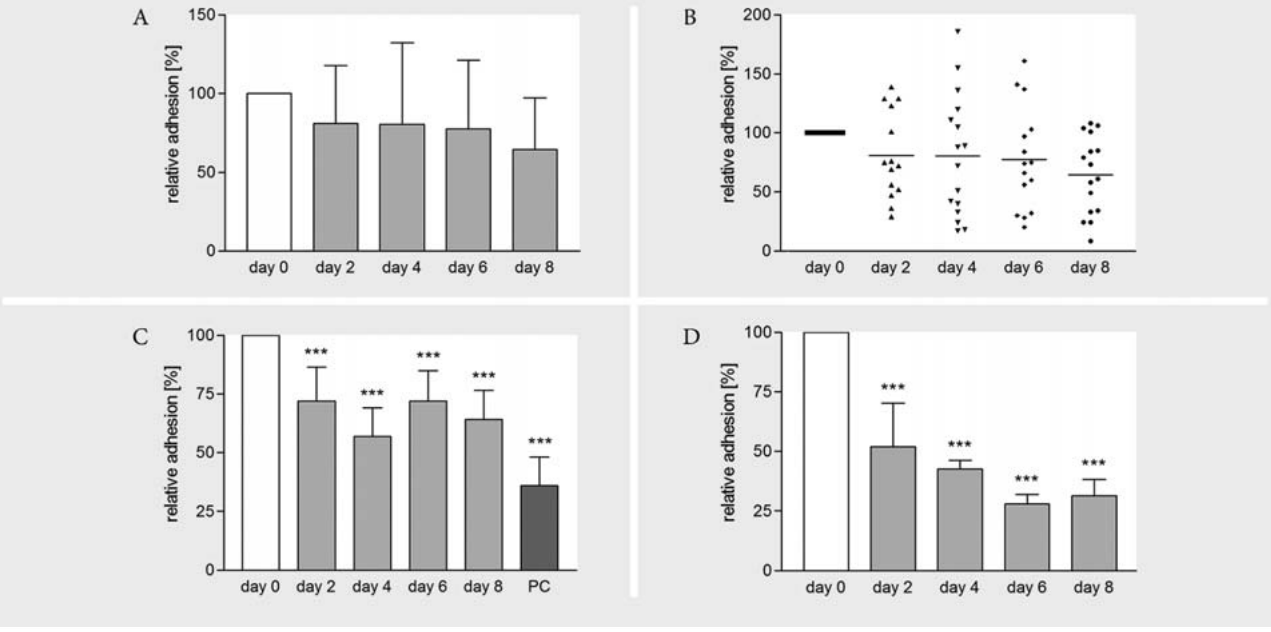
The cranberry dry extract (NutriCran 90S_06155, batch EK036155, extract-fruit ratio: 25 : 1, PACs (HPLC) > 2.7 %, supplier Naturex/Quiris Healthcare) from the fruits of *V. macrocarpon* is certified for the use as food product. The material was identified by HPLC analysis; voucher specimens are documented in the archives of University of Münster, Institute of Pharmaceutical Biology and Phytochemistry (IPBP-445). The extract is subsequently referred as CDE-Q.

Dereplication of cranberry dry extract CDE-Q (LC-MS)

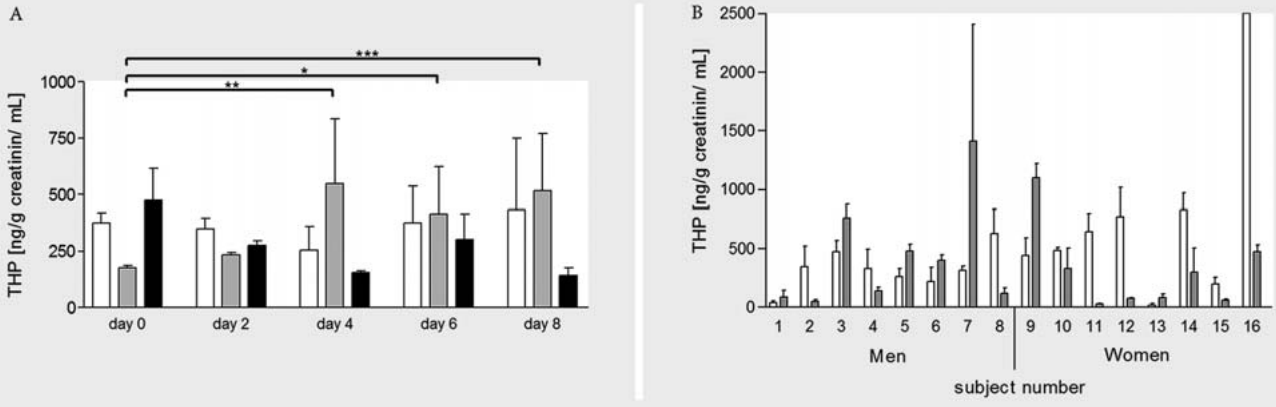
For the preparation of LC-MS samples, CDE-Q was dissolved in water to a concentration of 5 mg/mL. Chromatographic separation was performed on a Dionex Ultimate 3000 RS Liquid Chromatography System over a Dionex Acclaim RSLC 120, C18 column (2.1 × 100 mm, 2.2 µm) with a binary gradient (A: water with 0.1 % formic acid; B: acetonitrile with 0.1 % formic acid) at 0.4 mL/min; 0–9 min: linear from 5 to 100 % B; 9–15 min: isocratic at 100 % B; 15.0–15.1 min: linear from 100 to 5 % B; 15.1–20 min: isocratic at 5 % B. The injection volume was 2 µL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS over a wavelength range of 200–400 nm and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source in positive mode at 3 Hz over a mass range of *m/z* 50–1500 using the following instrument settings: nebulizer gas nitrogen, 4 bar; dry gas nitrogen, 9 L/min, 200 °C; capillary voltage − 4500 V; end plate offset − 500 V; transfer time 100 µs, prepulse storage 6 µs, collision energy 8 eV. MS/MS scans were triggered by AutoMS2 settings within a range of *m/z* 200–1500 using a collision energy of 40 eV and collision cell RF of 130 Vpp. Internal dataset calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10 mM solution of sodium formate in 50 % isopropanol that was infused during LC re-equilibration using a divert valve equipped with a 20 µL sample loop.

Manufacture of CDE-Q containing capsules for use in humans

CDE-Q, used for the biomedical study, was encapsulated (300 mg of extract per capsule) into cellulose capsules (size 1/white colored, batch-no. 10216036, WEPA) without addition of any further additives. The respective facility has been approved for manufacture of pharmaceuticals (Süd-Apotheke). The uniformity of mass of single-dose preparations was proven by a uniformity of mass test according to the requirements of European Pharmacopoeia [24]. Capsules were stored in plastic containers protected from heat and light at room temperature.



▶ **Fig. 7** Relative adhesion of UPEC strains UTI89 and NU14 to T24 bladder cells after pre-incubation with urine obtained from cranberry extract CDE-Q-treated volunteers (n = 16, 900 mg extract for 7 d). **A** Mean antiadhesive activity of individual urine test samples from 16 volunteers, UTI89; data origin from one experiment with n ≥ 2 technical replicates. **B** Antiadhesive activity of individual urine test samples from 16 volunteers, UTI89; data origin from one experiment with n ≥ 2 technical replicates. **C** Antiadhesive activity of pooled urines from 10 volunteers (5 male, 5 female), UTI89; data originate from 3 independent experiments with n ≥ 2 technical replicates. **D** Antiadhesive activity of pooled urines from 10 volunteers (5 male, 5 female), NU14; data originate from 3 independent experiments with n ≥ 2 technical replicates. *** $p < 0.01$. Values represent mean ± SD. PC: positive control, mannose 2.8 mM.



▶ **Fig. 8** Concentrations of THP in human urine samples obtained from cranberry extract CDE-Q treated volunteers (n = 16, 900 mg extract for 7 d). **A** THP concentration in pooled urine from 5 + 5 male/female volunteers (white bars), pooled urine from 8 male volunteers (gray bars) and 8 female (black bars); data originate from 2 independent experiments with n ≥ 2 technical replicates. **B** THP concentrations in individual urine test samples from 16 volunteers at day 0 (white bars) and day 8 (gray bars); * $p < 0.05$, ** $p < 0.01$. Values represent mean ± SD from 2 independent experiments.

Biomedical study design

The study protocol was approved by the ethics committee of the University of Münster (acceptance code: 2016-021-f-S, *V. macrocarpon*, 05.02.2016). Eight male and 8 female individuals gave written informed consent and volunteered to consume cranberry capsules over a 7-day period, with urine sampling at days 0, 2, 4, 6, and 8 for *ex vivo* studies. Exclusion criteria included antibiotic treatment 2 wk prior to and all along the study. Before starting the trial, the volunteers were instructed to abstain from consumption of any other products containing cranberry or phyto-

chemically or botanically similar fruits (especially from the plant family Ericaceae) 2 wk before and during the study. Each subject was asked to take 1 cranberry capsule 300 mg in the morning, afternoon, and evening regardless of food, equivalent to 900 mg CDE-Q per day for 7 d. Generally, the first midstream urine of the day was collected and used for functional and analytical investigations. A control urine sample (day 0) was collected prior to the consumption of the capsules.

The urine samples were filtered (0.22 µm pore size) and stored at − 20 °C until use. Five milliliters of each urine from 5 male and 5 female randomly chosen volunteers were pooled and named day 0 PU, day 2 PU, day 4 PU, day 6 PU, and day 8 PU. One milliliter samples of the respective urine from 8 female and from 8 male volunteers were pooled and named day 0/2/4/6/8 F PU (for women) or day 0/2/4/6/8 M PU (for men). These pooled urine samples were used for the adhesion assays as well as for quantitation of THP.

All urine samples obtained were tested on creatinin, pH, leukocytes, erythrocytes, sodium, potassium, chloride, bilirubin, glucose, and ketones. For investigating potential antiadhesive effects of the urine samples, an *ex vivo* antiadhesion assay was performed using 3 different *E. coli* strains and 2 different assay protocols: during co-incubation, fluorescent-labeled bacteria, T24 bladder cells, and urine samples were investigated; during pre-incubation, the bacteria were pre-incubated for 48 h with the respective urine samples.

The individual urine samples from the volunteers and the pooled urine samples obtained during the different treatment intervals were tested separately in the pre- and co-incubation protocols.

Cell culture and microbiology

T24 cells (ATCC HTB-4) represent a human epithelial bladder cell line, derived from the bladder carcinoma of an 82-year-old Swedish female [25]. These cells have been already demonstrated to be suitable for adhesion and invasion *in vitro* assays with UPEC [26] and were kindly provided by Prof. Straube (University of Jena, Germany). A498 cells (ATCC HTC-44) represent a human epithelial kidney cell line derived from the kidney carcinoma of a 52-year-old female [27] and were kindly provided by Dr. C. Hillgruber (Klinik für Hautkrankheiten, Münster, Germany).

Bacterial strains

E. coli strain 2980 (DSM 10791), provided by Prof. Straube (University of Jena), UPEC NU14 [28], a clinical cystitis isolate, UPEC UTI89 (NCBI: txid364106) [29], a clinical cystitis isolate, and UPEC CFT073 (NCBI: txid199310) [30], a clinical pyelonephritis isolate.

Bacteria from frozen stocks were cultivated for 48 h on UPEC agar supplemented with 0.2% $CaCl_2$. $CaCl_2$ is supposed to increase the expression of type 1 fimbria expression [31].

For urine culture, 1 CFU of overnight agar-grown bacteria was transferred to 9 mL of urine + 10% UPEC liquid medium (Tryptone 10 g, NaCl 8 g, glucose 1 g, yeast extract 1 g, water 1 L) in 50-mL tubes and incubated at 37 °C for 24 h. Afterward, 100 µL of the bacterial suspension ($OD_{640\,nm}$ = 0.1) were transferred into fresh urine with 10% liquid medium and again incubated at 37 °C.

Monitoring of bacterial growth in urine

One CFU of overnight agar-grown bacteria was transferred to 4.5 mL of urine + 10% UPEC liquid medium and incubated at 37 °C for 24 h. Bacteria were harvested by centrifugation and suspended in 1 mL liquid medium. Bacterial density was adjusted to an $OD_{640\,nm}$ of 0.2 in liquid medium and transferred in 10 µL aliquots into a 96-well plate. Additionally, 90 µL of fresh urine were added to the wells. The plate was incubated at 37 °C and bacterial growth was monitored by measuring the optical density every 30 min over a 6-h period and after 24 h at λ = 640 nm. Day 0 urine served as a control.

Adhesion assay with urine samples by quantitative flow cytometry

In general, FITC-labeling of UPEC and flow cytometric adhesion assay was performed as described by [2, 32]. Cells (1.25×10^5 cells/well) were seeded into 6-well plates and incubated at 37 °C/5% CO_2 until 90% confluence was reached (corresponding to 800 000 cells, after approximately 48 h of incubation). After this incubation period, the medium was removed and cells were washed once with PBS and once with DMEM (1 mL). All further steps with FITC-labeled E. coli ($OD_{640\,nm}$ 0.4, corresponding to 8×10^7 CFU/mL) were carried out under direct light protection. For adhesion experiments, a bacterial cell ratio of 100 : 1 was used. UPEC and T24 cells were incubated for 1 h (strain UTI89) or for 1.5 h (strain NU14) or for 2 h (strain 2980) at 37 °C. UPEC CFT073 and A498 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 1 mL trypsin/EDTA for 4 min at 37 °C. Trypsinization was stopped by addition of 2 mL DMEM + 10% FCS. The content of each well was transferred to tubes and centrifuged for 5 min at 450 g. The supernatant was discarded, and the cells resuspended in 700 µL of PBS. Fluorescence of the cell suspension was measured by flow cytometry. For data evaluation, 10 000 counts for each sample were used.

For quantitative in vitro flow cytometric adhesion assay with urine samples in co-incubation assay, 900 µL of urine samples were added per well, followed by addition of 100 µL of DMEM containing the labeled bacteria ($OD_{640\,nm}$ = 4).

For quantitative in vitro flow cytometric adhesion assay with urine samples in bacterial pre-incubation assay, the adhesion assay was performed similar to the assay described above with the following changes: bacteria were grown as described above in static culture at 37 °C for 48 h (24 h + 24 h) to induce type 1 pilus expression [33]. Subsequently, the bacteria were centrifuged at 8000 g for 10 min and washed with PBS, and the suspension was adjusted to an $OD_{640\,nm}$ of 8 in saline solution for FITC-labeling. After fluorescence-labeling, the density of bacteria was adjusted to an $OD_{640\,nm}$ of 4 in DMEM. One hundred microliters of the bacterial suspension and 900 µL DMEM were added to the T24 cells in 6-well plates, and the culture was incubated for 1, 1.5, or 2 h (according to the bacterial strain) at 37 °C. Finally, the bacterial adhesion was quantified by flow cytometry. Urine samples from day 0 from each volunteer served as untreated control. D(+)-Mannose (puriss. pa., Fluka) served as an in vitro positive control.

Invasion assay with urine samples

Invasion assay was performed according to [34]. Cells (1.25×10^5 cells/well) were seeded into 6-well plates and incubated at 37 °C/5 % CO_2 until 90 % confluence was reached (corresponding to 800 000 cells, after approximately 48 h of incubation). After this incubation, T24 cell culture medium was removed, and cells were washed once with PBS and once with DMEM. Bacteria were grown as described above in urine static culture at 37 °C for 48 h (24 h + 24 h) and afterward centrifuged at 8000 g for 10 min and washed with PBS, and the suspension was adjusted to an $OD_{640\,nm}$ of 4/mL in DMEM. One hundred microliters of the bacterial suspension and 900 µL DMEM were added to the T24 cells in 6-well plates and the culture was incubated for 2 h at 37 °C. Bacteria that did not interact during the incubation with T24 cells were removed by washing the T24 cells with 1 mL PBS/well 3 times. Subsequently, DMEM containing 100 µg/mL of the membrane-impermeable antibiotic gentamicin was added for 1 h at 37 °C to the samples in order to eliminate selectively extracellular bacteria. The antibiotic was removed by rinsing 3 times with PBS. Finally, cells were lysed by addition of 0.1 % Triton X-100, and the lysate was plated in 1 : 50 dilution onto UPEC agar and incubated overnight at 37 °C. Lysis released bacteria that have been already invasive before the addition of gentamicin and gave them the possibility to multiply on the agar plates. Anti-invasive activity was evaluated in the same dilution step for all samples by counting CFU. Bacteria grown in control urine (day 0) served as untreated control.

THP (uromodulin) assay

The concentrations of THP in the urines were quantified by Sandwich ELISA (Uromodulin Human ELISA Kit, Thermo Fisher Scientific). Urine samples were diluted 1 : 1000; 100 µL of the samples and different concentrations of THP reference standard were added. Incubation was performed for 2.5 h by gentle shaking (240/min) at room temperature. After rinsing, 100 µL biotinylated THP-antibody was added, followed by 1 h gentle shaking at room temperature. The supernatant was removed, and the wells were washed. One hundred microliters streptavidin HRP were added and the mixture was incubated for 45 min. The wells were rinsed and 3,3′,5,5′-tetramethylbenzidin was added and incubated for 30 min at room temperature by gentle shaking. The reaction was stopped by addition of 0.2 M sulfuric acid and the absorption of the resulting product was determined by at $\lambda = 450$ nm and a reference wavelength of $\lambda = 550$ nm. 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 4-parameter logistic curve fit for standard concentrations and then interpolation of sample absorbances.

Statistical analysis

Statistical results were obtained by use of GraphPad Prism statistics (version 3) (GraphPad Software). Results are expressed as the mean value (MV) ± standard deviation (SD). Data ($n \geq 3$) were processed by analysis of variance (one-way ANOVA). Subsequent *post hoc* test was conducted using the Tuckey test to determine the statistical significance of differences between mean values of 2 with each other compared groups. The level of significance was set to $p < 0.05$.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest. The study has been completely financed by intramural grants of the University of Münster, Germany. The cranberry extract has been supplied free of charge by Quiris Healthcare GmbH & Co.KG. The company did not have any influence on the study design, the experiments performed, and the evaluation of the data.

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