

In-vitro and in-vivo evidence of dose-dependent decrease of uropathogenic *Escherichia coli* virulence after consumption of commercial *Vaccinium macrocarpon* (cranberry) capsules

J.-P. Lavigne¹, G. Bourg¹, C. Combescure², H. Botto³ and A. Sotto¹

¹Institut National de la Santé et de la Recherche Médicale, ESPRI 26, Université de Montpellier 1, UFR de Médecine, ²Département de l'Information Médicale, Groupe Hospitalo-Universitaire de Carémeau, Nîmes and ³Service d'Urologie, Hôpital Foch, Suresnes, France

ABSTRACT

This study evaluated the antibacterial efficacy of the consumption of cranberry capsules vs. placebo in the urine of healthy volunteers. A first double-blind, randomised, crossover trial involved eight volunteers who had followed three regimens, with or without cranberry, with a wash-out period of at least 6 days between each regimen. Twelve hours after consumption of cranberry or placebo hard capsules, the first urine of the morning was collected. Different *Escherichia coli* strains were cultured in the urine samples. Urinary antibacterial adhesion activity was measured *in vitro* using the human T24 epithelial cell-line, and *in vivo* using the *Caenorhabditis elegans* killing model. With the *in vitro* model, 108 mg of cranberry induced a significant reduction in bacterial adherence to T24 cells as compared with placebo ($p < 0.001$). A significant dose-dependent decrease in bacterial adherence *in vitro* was noted after the consumption of 108 and 36 mg of cranberry ($p < 0.001$). The *in vivo* model confirmed that *E. coli* strains had a reduced ability to kill *C. elegans* after growth in the urine of patients who consumed cranberry capsules. Overall, these *in vivo* and *in vitro* studies suggested that consumption of cranberry juice represents an interesting new strategy to prevent recurrent urinary tract infection.

Keywords Adherence, *Caenorhabditis elegans*, cranberry, *Escherichia coli*, therapy, urinary tract infection

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INTRODUCTION

Each year, urinary tract infections (UTIs) account for >11 million physician visits in the USA and 2 million in France, and 3.5 million antimicrobial prescriptions [1,2]. *Escherichia coli*, the major pathogen involved in these infections, has developed new mechanisms of resistance against β -lactams and fluoroquinolones, which are the antimicrobial agents that are usually used to treat UTIs. A novel group of plasmid-mediated extended-spectrum β -lactamases, the CTX-M enzymes, has been reported. These enzymes hydrolyse broad-spectrum cephalosporins, with

higher levels of hydrolytic activity against cefotaxime than against ceftazidime, and are inhibited by suicide inhibitors [3]. The incidence of CTX-M enzymes has increased dramatically since 1995, with spread among other enterobacteria, in the community, and in most parts of the world [3]. These enzymes are now widespread in urinary *E. coli* strains, which have thus become multiresistant. Moreover CTX-M-producing strains generally have a high level of resistance to several other non- β -lactam antibiotic families, particularly the quinolones. It therefore seems essential to investigate potential new strategies for the prevention and/or treatment of UTIs.

In this context, it has been suggested that consumption of beverages containing cranberry juice is effective in preventing UTIs [4–6]. This presumed efficacy is related to the anti-adherent properties of cranberry juice [7]. Recently, the effectiveness of cranberry proanthocyanidins and

Corresponding author and reprint requests: A. Sotto, Institut National de la Santé et de la Recherche Médicale, ESPRI 26, Université de Montpellier 1, UFR de Médecine, CS83021, Avenue Kennedy, 30908 Nîmes Cedex 02, France
E-mail: albert.sotto@chu-nimes.fr

cranberry beverages against antibiotic-resistant *E. coli* has been described [8]. However, to date, few experiments have evaluated the effects of the consumption of cranberry juice on *E. coli* virulence against uroepithelial cells *in vitro*. Several clinical trials have investigated the efficacy of cranberry juice as prophylaxis against UTI [9], but the *in-vitro* and *in-vivo* effects of commercial cranberry juice preparations on *E. coli* virulence have not yet been documented.

Accordingly, the present study used a randomised double-blind trial to assess inhibitory activity in the urine of healthy subjects following consumption of commercial *Vaccinium macrocarpon* (cranberry) capsules on the adherence of resistant and susceptible *E. coli* strains to human uroepithelial cells. The virulence of the *E. coli* strains was also tested in a validated *in-vivo* nematode killing model [10].

MATERIALS AND METHODS

Healthy volunteers

Eight healthy female volunteers with a normal diet, aged 30–42 years, who belonged to the nurse population of the Foch Hospital, Suresnes, France, were included in a double-blind, randomised, placebo-controlled and crossover study. Administration of an antibiotic either in the 2-week period before the study or during the study, or pregnancy, were exclusion criteria. All volunteers gave informed consent to participate in the study.

Study protocol

The study was carried out using commercially available capsules of *V. macrocarpon* (cranberry) (Urell express; Pharmatoka, Rueil Malmaison, France) and capsules of placebo. The commercial cranberry capsules did not contain fructose, thereby allowing the effect of the proanthocyanidins to be evaluated. The placebo capsules contained colloidal silica, magnesium stearate, cellulose and gelatine. Each volunteer received three successive regimens with her evening meal. These comprised, in a random order: (i) three capsules of cranberry (108 mg), or (ii) three capsules of placebo, or (iii) one capsule of cranberry (36 mg) and two capsules of placebo. The dose tested in the present study corresponded to the dose used currently for prevention of UTI (an initial dose of 108 mg, and 36 mg daily thereafter). A wash-out period of at least 6 days between each regimen was allowed. Twelve hours after consumption of cranberry or placebo hard capsules, the first urine of the morning was collected, centrifuged at 4000 *g* for 15 min, sterilised by filtration (0.45- μ m filter), and stored at -20°C.

For the various assays, a collection of four uropathogenic *E. coli* strains, isolated previously from patients with symptomatic UTIs [10], was used: NECS20575 and NECS29787, which are strains with P-fimbriae *papG* and type 1 pili; NEC5,

a CTX-M-15-producing strain without P-fimbriae and type 1 pili; and NEC13, a TEM-3-producing strain with type 1 pili but no P-fimbriae.

In-vitro studies

Urinary bacterial anti-adhesion activity following product consumption was evaluated as described previously [1].

In a first assay, strains were subcultured overnight at 37°C to enhance production of P-fimbriae and type 1 pili. Undiluted urine from each regimen was then incubated for 20 min at 37°C with the different bacterial strains at a concentration of 10⁵ CFU/mL (corresponding to the bacterial concentration indicative of a clinical UTI), and this was followed by tests for the ability to agglutinate red blood cells using a mannose-resistant human red blood cell (HRBC) assay [11]. This activity was determined using a micro-haemagglutination test in 96-well round-bottomed plates in the presence of group A+ or O+ HRBCs newly drawn in citrate tubes. A 3% w/v suspension of HRBCs was added to each well containing dilutions of the bacterial/urine suspensions, and the microplate was agitated for 15 min. Each well was then evaluated microscopically for the presence or absence of haemagglutination. If the HRBCs in a well were not agglutinated, the urine in that particular well was considered to have cranberry metabolites with anti-adhesion activity. The final dilution at which agglutination suppression by the cranberry proanthocyanidins present in urine samples occurred was recorded. Wells containing only the erythrocyte suspension were used as negative controls. This rapid test evaluated the bacterial anti-adhesion activity of urine following consumption of Urell capsules.

In a second assay, *in-vitro* bacterial adherence experiments were performed with the human T24 epithelial cell-line (ATCC HTB-4) using an adaptation of the method of Di Martino *et al.* [4]. In brief, bacteria were grown overnight in human urine containing Luria-Bertani broth 5% v/v. Bacterial cells were harvested by centrifugation and resuspended at 10⁸ CFU/mL in McCoy's medium, added to a monolayer of the T24 cell-line, and incubated for 3 h at 37°C. After six washes with phosphate-buffered saline, the cells were fixed in methanol, stained with Giemsa 20% v/v, and examined microscopically under oil immersion. The average number of bacteria/T24 cell was determined by examining 100 T24 cells and was then recorded as the adhesion index. This index was expressed as a mean of at least four independent assays.

In-vivo model

The *Caenorhabditis elegans* infection assay was performed as described previously [10]. The Fer15 mutant line, which has a temperature-sensitive fertility defect, was used. The worms and *E. coli* strain OP50 (an avirulent control strain) were provided by J. Ewbank (CIML, Marseille, France). To synchronise the growth of worms, eggs were collected using the hypochlorite method. NGM plates were inoculated with a drop of an overnight culture of an *E. coli* strain and incubated at 37°C for 8–10 h. Plates were allowed to cool to room temperature and were then seeded with L4-stage worms (20–30/plate). The plates were then incubated at 25°C and scored each day for live worms under an MS5 stereomicroscope (Leica, Rueil-Malmaison, France). All four *E. coli* strains

were tested for their ability to kill *C. elegans*. At least three replicate assays, repeated on three occasions, were performed for each selected clone. A worm was considered to be dead when it no longer responded to touch.

Statistical analysis

Comparisons among the four *E. coli* strains, the three regimens and the two doses were evaluated using a two-way ANOVA procedure. The normality of the distribution was checked in each class by using a Shapiro–Wilks test. If an interaction was significant ($p < 0.05$), a one-way ANOVA was performed to determine the dose effect for each strain and the strain effect for each regimen. A Student–Newman–Keuls test was used to evaluate the statistical difference between each pair of groups. A Cox regression analysis was used to compare the entire survival curves in nematode killing assays, thereby allowing the timing of mortality between treatments to be compared. All analyses were performed using SAS/ETS v.9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Effect detected by in-vitro models

In total, 24 regimens were tested on four *E. coli* strains, representing 96 assays. For each group of strains, four independent trials were performed. The HRBC screening demonstrated that inhibition of agglutination occurred for the susceptible strains grown in urine collected from patients who had consumed three capsules of cranberry. This inhibition was dose-dependent (Table 1). The highest adherence index was obtained with the susceptible *E. coli* isolates grown in urine samples collected after consumption of three placebo capsules (Table 2). Three capsules of cranberry preparation caused a highly significant reduction in bacterial adherence to T24 cells as compared with placebo ($p < 0.001$). There was a dose-dependent decrease in bacterial adherence following cranberry intake. The adherence index obtained with bacteria grown in urine samples

Table 1. In-vitro inhibition of haemagglutination of human red blood cells (HRBCs)

Strain ^a	Susceptibility to β -lactams	Adhesins	HRBC assay (titre) ^b		
			3PI ($n = 8$)	1UE + 2PI ($n = 8$)	3UE ($n = 8$)
NECS20575	S	<i>papG+ / fimH+</i>	1/128	1/8	0
NECS29787	S	<i>papG+ / fimH+</i>	1/256	1/8	0
NEC13	TEM-3	<i>papG- / fimH+</i>	1/8	1/1	0
NEC5	CTX-M-15	<i>papG- / fimH-</i>	NA	NA	NA

3PI, regimen with three capsules of placebo; 1UE + 2PI, regimen with one cranberry capsule and two placebo capsules; 3UE, regimen with three cranberry capsules; NA, not applicable; S, susceptible.

^aThe results are representative of at least four independent trials for each strain.

^bResults indicate the final dilution at which agglutination suppression by the bacterial suspensions was observed.

collected after intake of three cranberry capsules was lower than that observed with one cranberry capsule ($p < 0.001$), even though a reduction in adherence was also noted with one cranberry capsule. The adherence to uroepithelial cells of the β -lactamase-producing NEC5 and NEC13 strains was also affected by the consumption of cranberry capsules. Finally, the NECS20575 and NECS29787 strains, which both produced PapG adhesin and type 1 fimbriae, adhered more efficiently to T24 cells than did the NEC5 strain, which did not produce these proteins, or the NEC13 strain, which produced only type 1 fimbriae ($p < 0.001$).

Reduction in virulence

Using the in-vivo model, the mean time at which 50% of the worms were killed (DL50) was increased significantly for strains NECS20575, NECS29787 and NEC13 grown in urine samples collected after cranberry intake, as compared with the same strains grown in urine samples collected after placebo intake (Table 3). All the worms infected with susceptible *E. coli* grown in urine containing cranberry metabolites were killed in 9 ± 0.25 days, which was significantly longer than the time required to kill worms infected with *E. coli* and grown in urine containing placebo (7.0 ± 0.5 days). This trend was also observed for the other strains, but the difference was not significant for NEC5, which does not produce adhesins. Cox regression analysis revealed that an infection with susceptible *E. coli* strains grown in urine containing cranberry metabolites increased the survival rate of worms by a factor of 4.03 ($p < 0.00001$).

DISCUSSION

Cranberry (*V. macrocarpon*) has been associated with the prevention of UTI for nearly 100 years [12]. A Cochrane Database Systematic Review has confirmed an interesting association between cranberry juice consumption and a decrease in the number of symptomatic UTIs [5]. However, there is no evidence that cranberry consumption can be used to treat UTI once an infection is present, and no beneficial effect was found in the prevention of UTI among patients with a neurogenic bladder [13] or among paediatric patients [14,15]. The safety of cranberry juice is considered

Table 2. Results of in-vitro experiments showing a reduction in adherence of *Escherichia coli* to T24 cells following cranberry intake by eight volunteers

Strain	Resistance to β -lactams	Adhesins	Adherence index \pm SD ^a			Decrease in bacterial adherence (%)		p ^b		
			3PI (n = 8)	1UE + 2PI (n = 8)	3UE (n = 8)	1UE + 2PI	3UE	3PI vs. 3UE	3PI vs. 1UE	1UE vs. 3UE
NECS20575	S	<i>papG+/fimH+</i>	22.49 \pm 2.47	13.78 \pm 2.63	5.61 \pm 2.90	38.7	75.1	<0.001	<0.001	<0.001
NECS29797	S	<i>papG+/fimH+</i>	22.32 \pm 2.22	14.40 \pm 2.54	5.76 \pm 2.20	35.5	74.2	<0.001	<0.001	<0.001
NEC13	TEM-3	<i>papG-/fimH+</i>	7.37 \pm 0.77	4.61 \pm 0.48	2.84 \pm 0.77	37.5	61.5	<0.001	<0.001	<0.001
NEC5	CTX-M-15	<i>papG-/fimH-</i>	4.84 \pm 0.26	3.42 \pm 0.49	1.70 \pm 0.46	29.3	64.9	<0.001	<0.002	<0.001
p ^c			NECS vs. NEC	<0.001	<0.001	0.002				

3PI, regimen with three capsules of placebo; 1UE + 2PI, regimen with one cranberry capsule and two placebo capsules; 3UE, regimen with three cranberry capsules.

^aThe index was calculated following at least four independent trials for each strain.

^bComparison using the same strain of different regimens.

^cComparison among strains for the same regimen.

Table 3. In-vivo kinetics of killing of *Caenorhabditis elegans* infected with *Escherichia coli* strains grown in urine samples following cranberry intake

Strain	Resistance to β -lactams	Adhesins	DL50 (days) \pm SD ^a		Death (days) \pm SD ^a		p
			3PI (n = 8)	3UE (n = 8)	3PI	3UE	
NECS20575	S	<i>papG+/fimH+</i>	3.21 \pm 0.36	5.43 \pm 0.22	7 \pm 0.25	9.5 \pm 0.25	<0.001
NECS29797	S	<i>papG+/fimH+</i>	3.03 \pm 0.22	5.11 \pm 0.40	6.5 \pm 0.25	9 \pm 0.25	<0.001
NEC13	TEM-3	<i>papG-/fimH+</i>	4.76 \pm 0.35	5.28 \pm 1.24	9 \pm 0.25	10 \pm 0.25	0.02
NEC5	CTX-M-15	<i>papG-/fimH-</i>	5.33 \pm 0.15	6.21 \pm 0.17	11 \pm 0.25	11.5 \pm 0.25	-

3PI, regimen with three placebo capsules; 3UE, regimen with three cranberry capsules; DL50, time at which 50% of the worms were killed.

^aThe results are means of at least four independent trials on different days and two independent trials on the same day for each strain.

to be excellent, although some adverse effects have been reported (e.g., a laxative effect [9] and urinary stones following the consumption of large amounts of cranberry juice over a long period [16]).

The effects of a daily dose of cranberry juice cocktail have been evaluated using ex-vivo and in-vivo clinical studies [8,17]. These studies revealed that 240–300 mL of cranberry juice could prevent recurrence of UTI [12], suggesting that a dose of <240 mL/day did not provide sufficient protection. No previous data are available concerning the dosage and efficacy of tablets or capsules. The present study used two bioassays to test the anti-virulence effect of commercial capsules of cranberry extract on four *E. coli* strains grown in urine samples collected after consumption of the capsules or placebo. Interestingly, cranberry intake inhibited the adherence of strains regardless of whether they produced P-fimbriae and type 1 fimbriae, as well as both antibiotic-resistant (CTX-M- and TEM-producing) and susceptible strains. Zafriri *et al.* [18] have previously studied the direct effect of cranberry juice and cranberry juice constituents on the adherence of *E. coli* expressing surface lectins of defined specificity for yeast, tissue culture cells, red blood cells and mouse peritoneal

macrophages. The present results confirmed the existence of a dose-dependent effect on the in-vitro adherence of *E. coli* to bladder epithelial cells, and demonstrated that the administration of three capsules (108 mg) of cranberry extract might represent an interesting alternative approach for the prevention of UTI, followed by a regimen of one capsule (36 mg) daily.

The cranberry extract comprises the polyphenolic part of the fruit that remains following removal of the other components of the berry (e.g., vegetable fibres and, more particularly, the acids and sugars of the fruit). The extraction process is not selective and all the polyphenols contained in the fruit are retained. During the initial step in the development of a UTI, *E. coli* produces hair-like fimbriae that protrude from the bacterial surface. These fimbriae carry adhesins that attach to receptors on uroepithelial cells [9]. Cranberry extract contains compounds, i.e., the proanthocyanidins, that inhibit *E. coli* adhesins and show strong inhibitory activity against P-fimbriae [11,18]. In the present study, the adherence index (i.e., the number of bacteria adhering to each cell) represents the activity of an oligomeric fraction of the proanthocyanidins belonging to type A. The adherence index following consumption of

placebo shows that P-fimbriae account for >85% of the overall adhesion, since net adhesion via type 1 fimbria was 2.5 ($AI_{NEC13} - AI_{NEC5}$, 7.3 – 4.8), while that of P-fimbriae and type 1 fimbriae was 17.5 ($AI_{NECS} - AI_{NEC5}$, 22.3 – 4.8). Moreover, it seems that strains that lack type 1 and P-fimbriae adhere via an adhesin whose synthesis is inhibited by urine containing cranberry metabolites, suggesting that cranberry extract affects various fimbriae, but particularly P-fimbriae.

The *C. elegans* model has been used previously to study bacterial pathogenicity [19], and it has been demonstrated that the ability to kill worms can be used to evaluate the virulence of uropathogenic *E. coli* [10]. Uropathogenic *E. coli* strains are only pathogenic after ingestion by *C. elegans*, often exerting their effect in the anterior part of the worm's intestine, where they may establish an intestinal infection. The present study revealed that reduced ability of uropathogenic *E. coli* strains to kill worms correlates with the consumption of cranberry capsules. Bacteria grown in the urine of individuals who had consumed cranberry capsules were unable to adhere to the worms, resulting in reduced killing of the worms. However, the possibility that worm-specific virulence factors other than adhesins might be involved cannot be excluded.

One of the main limitations of this study was that cranberry proanthocyanidins in urine samples were not measured. To date, few pharmacokinetic studies, and no in-vivo studies, have been performed on proanthocyanidins because of the structural complexity of these molecules and the absence of commercial standards. Research has shown that proanthocyanidins reach the urine of humans and mice after consumption of cranberry juice; thus oral delivery of ^{14}C -labelled grape proanthocyanidins to rats resulted in 19% of the dose being excreted in the urine, and 45% in the faeces [12]. Activity increased continuously in a regular progression, peaking at 4–6 h post-consumption, and persisted in the urine for at least 8 h, suggesting potential protection against bacterial attachment to the uroepithelium during this period [20]. In the present study, it was assumed that cranberry proanthocyanidins reached the urine of individuals and formed the active ingredient in the various tests. However, the possibility of other explanations, e.g., an enhancement of the innate immune system, cannot be excluded.

Nevertheless, this approach provides an interesting new strategy for the prevention of recurrent urinary tract infections. With the growing resistance to antibiotics, cranberries can be viewed as a potentially useful non-pharmaceutical prophylactic remedy.

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