Effect of cranberry drink on bacterial adhesion in vitro and vaginal microbiota in healthy females

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Introduction/objective: *Cranberries have been shown to produce urinary metabolites that influence uropathogen adhesion and prevent urinary tract infections. This study was designed to determine if consuming reconstituted, unsweetened cranberry drink from extract retained its bioactive properties by reducing uropathogen adhesion without adversely affecting urinary calcium, magnesium and the vaginal microflora.*

Materials and methods: *A randomized crossover study was undertaken in 12 healthy women consuming reconstituted unsweetened cranberry drink, CranActin or water. The urine was collected at 4 hours and 1 week of consumption and evaluated for antiadhesive properties and urinary pH, calcium and magnesium. Vaginal swabs were collected after 1 week of treatment to assess the vaginal microbiota by DGGE.*

Results: *The resultant urine produced by subjects who consumed 500 ml reconstituted cranberry extract twice per day, significantly reduced the adherence to epithelial cells of P-fimbriated uropathogenic Escherichia coli and showed a tendency towards significance for two E. coli strains expressing fimbriae and an Enterococcus faecalis isolate. The cranberry drink treatment did not alter urinary pH, but reduced calcium and magnesium concentrations compared to water, although not to statistical significance. The reconstituted cranberry drink had no apparent detrimental effect on the vaginal microbiota. However, consuming twice daily resulted in an apparent loss of a potential pathogen from the vagina in 42% subjects.*

Conclusions: *The present findings suggest that reconstituted cranberry drink may retain the ability to reduce the risk of UTI by inhibiting pathogen adhesion while not detrimentally affecting urinary pH or vaginal microbiota, or the risk of calculi.*

Key Words: vaginal microbiota, cranberry drink, urinary tract infections, fimbriae adhesins, *Escherichia coli, Enterococcus faecalis*

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Introduction

Urinary tract infections (UTI) afflict millions of women each year. Many episodes are recurrent, and all have an adverse impact on quality of life. The most common causes of uncomplicated community acquired UTIs are uropathogenic *Escherichia coli* (UPEC), followed by *Enterococcus faecalis* and *Klebsiella pneumoniae*.[1] An important stage in the infection process is adhesion to the bladder epithelium. UPEC strains express a number

of adhesins, particularly Type1-and P- fimbriae that have tropism for glycolipids enriched on the cell surface of the bladder epithelium and kidneys. Type 1 fimbriae adhere to mannose residues and are expressed by most *E. coli*, including 90% of fecal isolates. P-fimbriae are primarily found in UPEC strains causing pyelonephritis, and there are three different isotypes (GI, GII and GIII) that adhere to various globoside residues distributed in the bladder and kidneys.[2] Disrupting the adhesins would alter the adhesion process and thus the progression of UTI.

Antibiotics are generally successful in treating UTI, but side effects occur in approximately 30% of cases, and drug resistant uropathogens are increasing. These problems, and a consumer trend to using natural products, have led to the development of alternative functional foods to reduce the risk of UTI. Juice from cranberries (*Vaccinum macrocarpon*) has one of the longest traditions as a natural therapy for urinary tract health, and in recent times, human studies have suggested that it can reduce the occurrence and symptoms of some episodes of UTI.[3,4] *In vitro* studies have demonstrated that a high molecular weight fraction of cranberry extracts prevents adhesion of pathogens to cells and biomaterials. Cranberries along with various fruits are especially rich in complex polyphenolic compounds, however only cranberries are uniquely enriched with proanthocyanidins (PACs).[5] These inhibit adhesion of fimbriated *E. coli* to tissue cells *in vitro*,[6,7] while the inhibition of Type 1 fimbriae adhesion has been attributed to fructose.[8,9] Cranberries have been reported to decrease adherence of *E. coli* to glass, implying that the effect is not solely due to interference with glycolipid receptors sites.[10,11]

Anthocyanins have been found excreted in significant quantities in the urine of individuals consuming cranberry products, suggesting that PACs are likewise excreted.[12] A previous study evaluating antiadhesive properties of urine from individuals consuming cranberry juice found a dose dependant decrease, however they did not assess the individual adhesins.[13] Bladder cells from spinal cord injured patients consuming cranberry juice, are reportedly less receptive to bacterial adhesion and biofilm formation,[14] and they can inhibit and kill staphylococci.[15]

The exact nature of any antiadhesive compound(s) present in urine remains unknown, however they are believed to be PACs or their metabolites.[11] Ingested anthocyanins were excreted at lower levels than when ingested as juice mixtures, suggesting other components influence urinary levels.[12] We hypothesized that reconstituted cranberry drink that contains PAC retains its antiadhesive properties for uropathogenic bacteria in the urine of healthy individuals without disturbing the normal vaginal *Lactobacillus* population. The present study tested this *in vitro* and *in vivo* using urine from volunteers who consumed reconstituted unsweetened cranberry drink from cranberry extract.

Materials and methods

Clinical study

Twelve healthy women, aged 19-45 years, volunteered to take part in a randomized crossover study. The subjects had no history of chronic UTI, no UTI in the previous year, and were currently not taking antibiotics or any anti-UTI supplements. The study excluded subjects with urinary tract abnormalities, requiring catheterization for micturition and those who were pregnant, diabetic or had known allergies to berry juices. The study was approved by the University of Western Ontario Human Ethics Review Board (12064E) and registered with Clinical trials ID#R-06-704.

During the trial, subjects maintained their normal diet, consumed water at libitum but avoided cranberry or other berry fruits or juices, vitamin C supplements and probiotic yogurts. The study compared mineral water, CranActin together with mineral water and a cranberry drink from reconstituted cranberry extract in mineral water. The four study regimes of 7 days (1 week) each consisted of: a) 500 ml bottled mineral water twice daily; b) four capsules of CranActin (Solaray, 400 mg cranberry AF extract, 30 mg vitamin C) with 500 ml bottled mineral water twice daily; c) 500 ml bottled reconstituted cranberry drink once daily and 500 ml bottled mineral water once daily; or d) 500 ml bottled reconstituted cranberry drink twice daily. Participants were randomly assigned to begin with a, b, c, or d regiment. Between each study regimen, a washout period of 7 days was incorporated during which subjects returned to their normal diet with no added beverage or food supplements, and no cranberry fruit or juice.

A single midstream urine sample was collected 4 hours after the initial consumption of the reconstituted cranberry drink to evaluate any antiadhesive effects acquired from a single dose. Subsequently, first morning midstream urine was collected on days 0, 8, 15, 22, 29, 36, 43, and 50 to evaluate antiadhesive properties of the urine. The urine samples were filter sterilized (0.22 µm) and stored at 4°C and were used within 5 days of collection.

A 24 hour urine sample was collected after 3-4 days of each phase and tested for pH, oxalates, calcium and magnesium, indicators of increased risk of kidney stone formation.

Vaginal swabs were collected day 0, 8, 22, 36, and 50 and evaluated by density gradient gel electrophoresis (DGGE) to determine the effect of cranberry products on the vaginal microbiota.

TABLE 1. **Bacterial strains used in this study**

Bacteria	Plasmid	Description	Refs
E. coli GR12	none	Clinical isolate – uropathogen; expresses P-fimbriae GII and Type 1 fimbriae	
E. coli HB101	none	K12, afimbriated	
E. coli HB101	pDC1	K12, expressing P- fimbriae GII (pACYC:pap_{1A2})	16
E. coli HB101	pPap5	K12, expressing P- fimbriae GI (pBR322:pap_{J96})	16
E. coli HB101	pSH2	K12, expressing Type I fimbriae (pACYC184:fim_{J96})	
E. faecalis 1131	none	Clinical isolate	11

Bacteria and growth conditions

The study strains are listed in Table 1 and include *E. coli* expressing different adhesins and *E. faecalis*. The *E. coli* were grown on different media to optimize adhesion expression, together with the appropriate antibiotics (carbenicillin 100 ug/ml, chloramphenicol 30 ug/ml). *E. coli* strain was grown on brain heart infusion (BHI, Difco) agar (Type 1 fimbriae) or Lauria Bertani (LB, Difco) agar (P-fimbriae, control and UPEC). *E. faecalis* was cultivated on sheep blood agar. Bacterial cultures were grown on agar plates overnight at 37˚C and suspended in phosphate buffered saline (PBS, pH 7.4). The bacterial suspension was then added to urine to an optical density of 0.7-1.0, depending on the bacterial strain, and incubated at 37˚C for 1 hour. They were evaluated for adhesion by hemagglutination or binding to human bladder cells.

Cell adhesion assay

T24 human bladder epithelial cells (ATCC HTB-4) were grown in RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (Gibco) at 37˚C and 5% CO_2. Bacteria treated in urine were combined with an equal volume of tissue culture media lacking antibiotics (approx 1 x10^8 CFU/well) and added to of a 24-well plate containing a confluent layer of T24 bladder cells. The plates were centrifuged to ensure bacterial contact with the cells and incubated for 2 hours. Triplicate samples were tested, with one used to determine total bacterial counts and two washed five times with PBS to evaluate adherent bacteria. Bacterial counts were determined by lysing the tissue cells with 5% Triton X-100 and serial dilution plating of the bacteria for colony forming units (CFU).

Hemagglutination

E. coli incubated in sterile morning urine for 1 hour were combined with 8% human erythrocytes on an ice chilled glass plate and hemagglutination was evaluated as either -, +/-, or + representing no agglutination, partial agglutination or full agglutination. *E. coli* HB101 was a negative control since it did not express fimbriae, had no agglutination, and was therefore not included in the statistical analysis. Data were analyzed using Pearson chi-square (SPSS v14).

Denaturing gradient gel electrophoresis (DGGE)

The vaginal bacterial flora was assessed after 1 week of consumption of all four regimes, using DGGE. Total DNA was extracted from the vaginal swabs using Instagene matrix (Biorad) according to manufacturers instructions and stored at -20˚C. Bacterial DNA was amplified by polymerase chain reaction (PCR) using either the Eubacterial primers with GC clamps or the *Lactobacillus* primers with GC clamp. The PCR products were subjected to DGGE for separation and select bands were sent for sequencing. Each sequence was compared to the NCBI database for identification. In addition to DGGE, *E. coli* specific primers were used to determine if *E. coli* were also present in the vagina using PCR and agarose gel electrophoresis.

Results

Agglutination and adhesion of bacteria to tissue cells

All of the recombinant strains of *E. coli* and the UPEC GR12 have been previously shown to agglutinate human erythrocytes.[16] As a control for the hemagglutination assay the *E. coli* cultures were all tested for agglutination prior to treatment with urine and only those exhibiting full agglutination were used. The bacteria were treated with subject's urine and as all tests exhibited either full or partial agglutination with little gradient, the data were collapsed into two categories of "full" and "partial" agglutination. The findings are shown in Figure 1. There was no statistically significant difference in agglutination scores between the treatments and

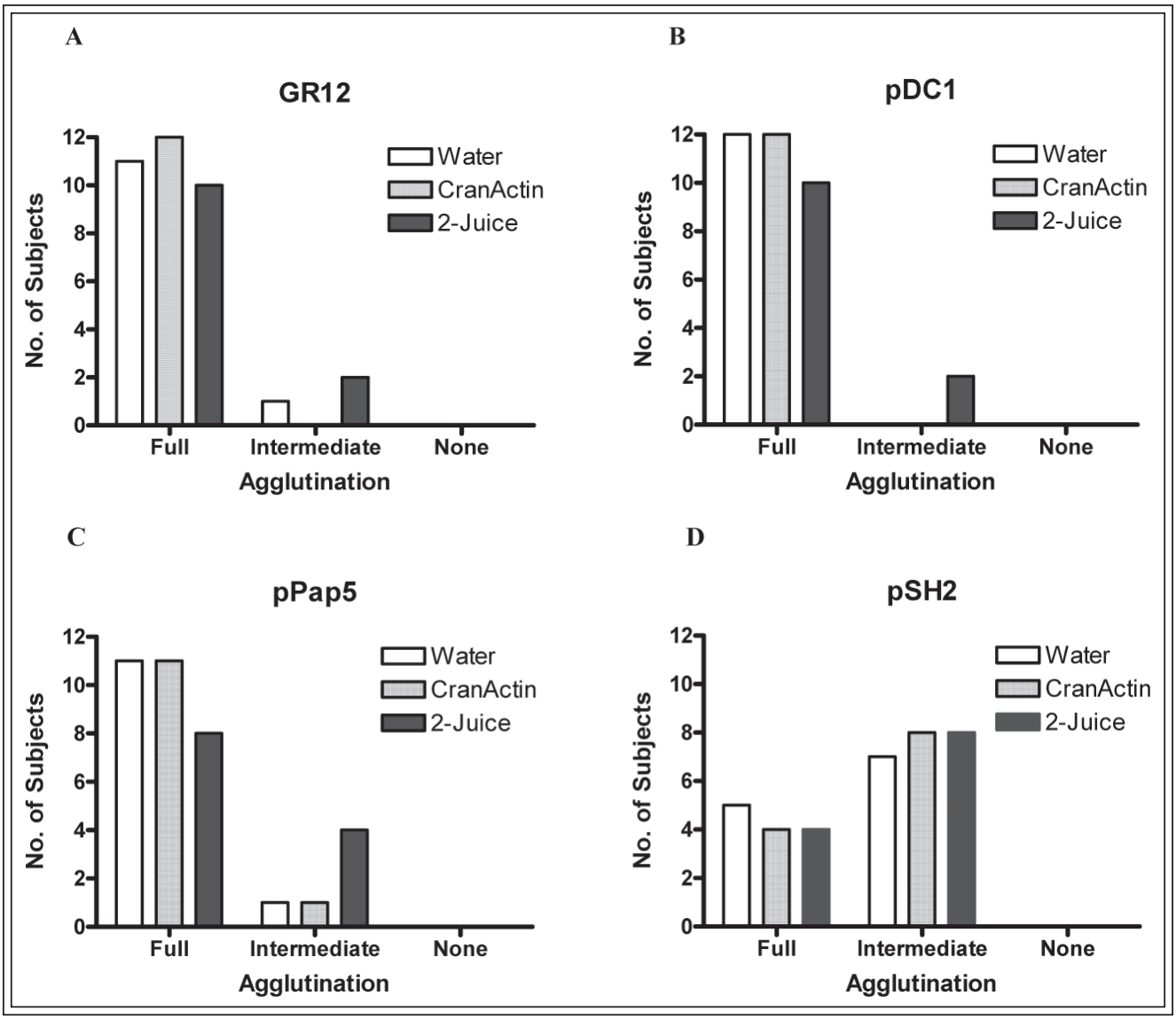

Figure 1. Hemagglutination of *E. coli* treated with urine from participants that consumed water or cranberry drink. A) UPEC GR12; B) HB101/pDC1 (P-fimbriae, G-II); C) HB01/pPAP5 (P-fimbriae, G1) and D) HB101/pSH2 (Type 1 fimbriae). No significant effect on agglutination was observed by any treatment (2-sided Pearson Chi-square).

none totally eliminated agglutination in any strain. However, the strains carrying P- fimbriae tended to show intermediate agglutination more often with reconstituted drink than with water and CranActin. The recombinant strain with Type 1 fimbriae had more intermediate than full agglutination for all treatments.

The results in Figure 2 show that taking two doses of reconstituted cranberry drink tended to decrease bacterial adhesion when compared to water for all the bacteria except the strain expressing PapGI adhesin of P-fimbriae. The statistical analysis, shown in Table 2, indicate that urine from subjects who consumed reconstituted drink twice per day inhibited all bacterial adhesion except *E. coli* expressing PapGI adhesin. Whereas, consuming CranActin did not show trends towards reducing bacterial adhesion except in the UPEC GR12 strain and the afimbriated strain. The single dose of reconstituted cranberry drink per day did not significantly inhibit adhesion of any bacterial strains tested.

Urinalysis of subjects consuming cranberry drink

There were no significant pH changes following consumption of either reconstituted cranberry drink or CranActin (Mann-Whitney U test, significance level = 0.1), Table 3. A 24 hour collection of urine would not detect

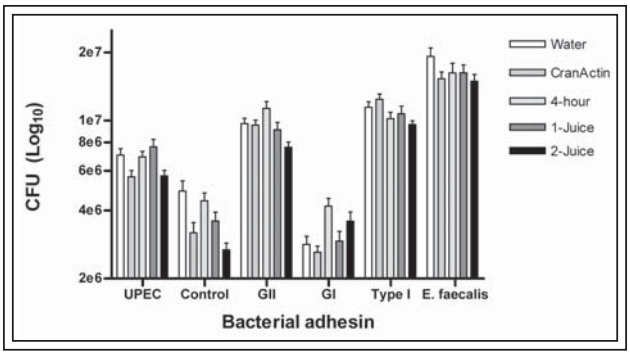

Figure 2. Average number of bacteria per tissue cell after treatment with urine from volunteers taking mineral water, CranActin or 1 and 2 doses of reconstituted cranberry drink for 1 week or after a single dose 4 hours). Error bars = SD.

fluctuations in urine pH at different times of the day, or following the consumption of drink, thus, pH was also measured on the first morning collection after 7 days of treatment and 4 hours after a single dose of drink. Again, there was no significant differences at 7 days, but urine collected at 4 hours was associated with lower pH compared to water, albeit not statistically significant at 90% level of confidence (p = 0.101, Mann-Whitney U test).

The average concentrations of Ca^{++}, Mg^{++} and oxalates were within the normal healthy range, with some insignificant (significance level = 0.1) fluctuations, Table 3.

Influence of cranberry products on the vaginal bacterial microbiota

The healthy vagina of premenopausal women contains *Lactobacillus iners* and species from the *Lactobacillus acidophilus* complex, which is composed of genetically similar rRNA species including *L. acidophilus, L. crispatus, L. amylovorous, L. gallinarum, L. gasseri* and *L. johnsonii* that cannot be differentiated by standard DGGE. With the exception of one, all subjects had either an organism from the *L. acidophilus* complex or *L. iners* present at all sample times, Table 4. One subject who did not have *Lactobacillus* sp. present in the vaginal swab, had *E. coli* and an uncultured *Prevotella* throughout the study, indicative of bacterial vaginosis. The consumption of cranberry drink did not alter this profile.

There was no difference in the recovery of *E. coli* from the vagina of subjects who drank water and reconstituted cranberry drink, however, more strains were recovered during CranActin consumption from 17% (in water) to 33% (CranActin), Table 4. In five subjects (42%) who consumed water or CranActin and three subjects (25%) who consumed reconstituted cranberry drink, a potential pathogenic organism (*Shigella/E. coli* clone, *Gardnerella*, unknown organism) was detected. In one case (8%), a potential vaginal pathogen, *Gardnerella* was detected during reconstituted cranberry drink consumption and not during water or CranActin use.

Discussion

In this study, *in vitro* antiadhesive properties against a clinical isolate of uropathogenic *E. coli*, plus strains expressing P-fimbriae PapGII and Type 1 fimbriae, and an *E. faecalis* isolate, were shown to be present in urine from healthy female volunteers who consumed 500 ml of reconstituted cranberry drink twice daily, when compared to mineral water. Two doses per day of the reconstituted cranberry drink were either better or comparable to 8 capsules of CranActin per day. Fructose and vitamin C were previously found to have minimal or no observable antiadhesive effects.[12]

TABLE 2. **Statistical analysis of bacterial adhesion**

Bacteria	**Phenotype**	**p-value***	
		2-cranberry drink versus water	**CranActin versus water**
E. coli GR12	Type 1 and P fimbriae (PapGII)	0.06	0.02
E. coli HB101	afimbriated	0.10	0.07
E. coli HB101/pDC1	P-fimbriae PapGII	0.01	0.96
E. coli HB101/pPap5	P-fimbriae PapGI	0.13	0.87
E. coli HB101/pSH2	Type I fimbriae	0.07	0.39
E. faecalis 1131		0.07	0.20

*All analysis done using the Mann-Whitney U test (significance level = 0.1)

TABLE 3. **Urine analysis of subjects in the study**

Treatment	pH			Calcium	Magnesium	Oxalates
	4 h	7 d	24 h	mM	mmol/d	µmol/d
Water	ND	6.1 (0.4)	6.1 (0.4)	4.8 (2.8)	3.6 (1.7)	221.2 (81.5)
CranActin	ND	5.9 (0.6)	6.1 (0.5)	3.7 (2.7)	4.2 (1.4)	231.2 (77.2)
1 drink/day	5.7 (0.5)	6.0 (0.5)	6.1 (0.5)	3.8 (3.1)	3.6 (1.2)	253.3 (141.2)
2 drink/day	ND	5.8 (0.4)	6.0 (0.4)	3.8 (2.9)	3.3 (1.8)	247.2 (121.9)

Note: (± SD); ND = not determined

Inhibition of receptor mediated adhesion was not observed, as shown by the lack of significant inhibition of hemagglutination by urine of participants taking cranberry products and drink. This could be due to qualitative nature of the assay. An analysis of adhesion mechanisms by Liu et al showed that interference was mediated, in part, by nonspecific interactions.[17] It is possible that metabolites alter the fimbriae conformation, and thus reduce nonspecific adhesion. It may be that urinary metabolites weaken the fimbriae binding interaction rather than totally inhibit their activity, therefore, *in vivo*, the urine flow might more easily eliminate the bacteria from the bladder. Human urine normally contains some levels of manno-oligosaccharides known to inhibit Type 1 fimbriae, and this may explain the lower agglutination exhibited by organisms expressing Type 1 fimbriae, even in the absence of added sugar.[18]

Cranberry juice has been thought to prevent stone formation due to its ability to lower urinary pH and citric acid content, which increases urinary citrate. However, a recent report suggested that it may increase the risk of calcium oxalate and uric acid stone formation, but reduce the risk of brushite stones.[19] Alternately, other reports demonstrated no significant changes in urine pH after consuming cranberry juice or cranberry products.[11,13] In our study, there was no evidence that the reconstituted cranberry drink significantly decreased urinary pH or increased the Ca^{++} or Mg^{++} levels, but rather, the data showed a reduction in these minerals. There was a small elevation within the normal range in oxalate composition of the urine. A longer and more focused clinical study would be needed to assess that the reconstituted cranberry drink decreases Ca^{++} or Mg^{++} in patients predisposed to urolithiasis.

A previous study showed that cranberry juice did not alter nasopharyngeal bacterial population in children, however the authors suggested that fecal flora was altered by detecting a significant change in fatty acid composition.[20] *E. coli* pathogens causing UTI ascend from the rectum along the perineum to the vagina and into the bladder. Some pathogens may reside in the vagina if the natural flora is disrupted, predisposing the woman to UTI by altering adhesion of natural microbial residents. Vaginal swabs evaluated for *Lactobacillus* sp and *E. coli* from the volunteers after consuming the reconstituted cranberry drink showed no detrimental effects on the resident *Lactobacillus* population of the vagina. In 42% of cases (5 subjects), there was an apparent loss of potential pathogens following consumption

TABLE 4. **Incidence of bacteria in vaginal swabs**

Conditions	Water		CranActin		1 Drink		2 drink	
No. of subjects	Present	Absent	Present	Absent	Present	Absent	Present	Absent
Lactobacillus sp	11	1	11	1	11	1	11	1
L. acidophilus complex	8	4	8	4	8	4	8	4
L. iners	5	7	4	8	4	8	4	8
E. coli	2	10	4	8	1	11	2	10
Other[a]	5	7	4	8	3	9	3	9

[a]detected uncultured bacteria including *Gardnerella* and *Prevotella* sp. and *Shigella* / *E. coli clone*

of reconstituted unsweetened drink twice daily. This suggested a selective antiadhesive property of cranberry metabolites. This small study on healthy volunteers provides a valuable foundation for future studies on women with recurrent UTI, to determine whether reconstituted cranberry drink containing PAC can reduce the incidence of UTI.

In conclusion, reconstituted unsweetened cranberry drink retained the antiadhesive properties of natural cranberry products in the urine against uropathogenic bacteria when evaluated *in vitro*, with no detectable major adverse impact on the vaginal microbiota. □

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