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PAPER
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A randomized, double-blind, placebo-controlled pilot study to assess bacterial anti-adhesive activity in human urine following consumption of a cranberry supplement†

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Urinary tract infections (UTIs) are one of the common bacterial infections treated with antibiotics. The North American cranberry is recommended for prophylaxis in women with recurrent UTIs as a nutritional alternative. The ability of cranberry components and their metabolites to inhibit adhesion of uropathogenic *Escherichia coli* (*E. coli*) is an important mechanism by which cranberry mitigates UTIs. The objective of this study was to evaluate urinary anti-adhesion activity against type 1 and P-type uropathogenic *E. coli* after consumption of cranberry +health™ cranberry supplement (cranberry chew). In this randomized, double-blind, placebo-controlled, crossover design pilot trial (*n* = 20), subjects consumed two cranberry or placebo chews, one in the morning and one in the evening. Clean-catch urine samples collected at the baseline and post-intervention (0–3, 3–6, 6–9, 9–12, 12–24, 24–30, 30–36 h) were tested for anti-adhesion effects with a mannose-resistant human red blood cell hemagglutination assay specific for P-type *E. coli*, or a T24 cell line model for type 1 *E. coli*. Urinary anti-adhesion activity against P-type *E. coli* after consumption of the cranberry chew was significantly greater (*p* < 0.05) than that observed with placebo chew at all time points except 24–36 h. *Ex vivo* anti-adhesion effects on type 1 *E. coli* were greater (*p* < 0.05) after cranberry chew consumption than placebo chew at 3–6 and 6–9 h urine collections. In conclusion, consumption of cranberry +health™ cranberry supplement exhibited greater *ex vivo* urinary anti-adhesion activity compared to placebo, suggesting that it may have the potential to help promote urinary tract health.

1. Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections with about 150 million cases occurring globally every year.[1,2] It is estimated that over 50% of women experience at least one infection in their lifetime; however, recurrence rates are high (30–50%) with a third of these women reporting recurrent episodes within 6 months.[3] Treatment of UTIs often requires the use of antibiotics, but frequent use of antimicrobials has caused significant public health concerns about antibiotic resistance and emergence of multidrug resistant organisms. Nutritional alternatives such as cranberry and cranberry products to maintain urinary tract health have been recommended for women with recurrent UTIs.[4–6] Two recent meta-analyses supported the beneficial effects of cranberry consumption on reducing risk of UTIs. Fu *et al.* found that cranberry reduced the risk of UTIs by 26% (pooled risk ratio: 0.74; 95% CI: 0.55, 0.98; I^2 = 54%),[4] and Luis *et al.* showed that cranberry products significantly reduced the incidence of the infections with a weighted risk ratio of 0.675 (95% CI 0.5516–0.7965, *p* < 0.0001) for a heterogeneous population.[5] However, inconsistent evidence is still being observed in some clinical trials[7] and may be attributed to various factors including inappropriate subject population, high withdrawal rate or poor compliance, lack of standardization of cranberry bioactive components in administered treatment products and suboptimal dosing.

Uropathogenic *Escherichia coli* (UPEC) is the most common pathogen causing 75–95% of uncomplicated cystitis and pyelonephritis.[8] UPEC expresses a broad spectrum of virulence factors with pathogenicity being determined predominantly by surface adhesive molecules. To initiate an infection, uropathogens first utilize their fimbriae or pili to adhere to the uroepithelial cell receptors, leading to bacterial proliferation.[9]

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Three UPEC fimbrial types are associated with most infections. UPEC expressing type 1 fimbriae primarily bind to uroplankin Ia receptors causing the majority of acute cystitis.[10] P-fimbriated UPEC plays an important role in the pathogenesis of ascending UTIs and pyelonephritis[11] and UPEC expressing F9 fimbriae are associated with binding to chronic and inflamed bladder epithelium.[12] Extensive research has been conducted to investigate bioactive components in cranberries that are responsible for the anti-adhesion activity. Previous studies have shown that cranberry polyphenolic compounds such as proanthocyanidins,[13,14] flavonols and their glycosides (*e.g.* myricetin, quercetin),[15,16] condensed tannins (*e.g.* procyanidins, prodelphinidin),[15,17] and phenolic acids (*e.g.* benzoic acid)[18] were able to inhibit the *in vitro* adherence of P-type *E. coli* to the glycoprotein receptor on epithelial cells or red blood cell analogues. Certain parent phenolic components in the cranberries and gut microbial derived phenolic catabolites (*e.g.* phenols, valerolactone and phenolic acids) showed anti-adhesion potential. Two of the most abundant metabolites (hippuric acid and α-hippuric acid) after cranberry consumption exhibited anti-adhesion effects on P-type UPEC at physiologically relevant concentrations (100 to 500 μM).[18] In addition, metabolites such as catechol, vanillic acid, phenylacetic acid and 3,4-dihydroxyphenylacetic acid were also able to inhibit the adherence of P-type UPEC to uroepithelia cells in a concentration-dependent manner.[18] Moreover, the gut microbial degradation products of cranberry PACs, valerolactone and its derivatives exhibited anti-adhesion properties against P-type UPEC at a concentration of 100 μM, indicating their potential biologically relevant activity in urine.[19] The anti-adhesion activities of cranberry-derived metabolites suggest that their presence in the urine could be responsible for the further reduction of bacterial colonization and pathogenesis of UTIs.

Most *ex vivo* studies using urine from subjects after cranberry consumption have been focused on anti-adhesion effects on UPEC expressing P-fimbriae.[20–23] Only a few studies have investigated the inhibitory effects of cranberry consumption on UPEC expressing type 1 fimbriae.[24,25] The objective of this study was to investigate the urinary anti-adhesion activity against both type 1 and P-fimbriae UPEC in healthy individuals following the consumption of cranberry products in a pilot trial. We performed this randomized, double-blind, placebo-controlled, crossover design pilot trial to investigate whether the cranberry chew (cranberry +health™ cranberry supplement) would provide strong *ex vivo* anti-adhesion effects in the urine compared to a placebo chew.

2. Experimental

2.1 Clinical study design

The pilot study was approved by the Institutional Review Board at Rutgers University. Twenty healthy male and female subjects without a history of current or recurrent urinary tract infections, urinary disorders, or antibiotic use within the last six months were recruited for this randomized, double-blind, placebo-controlled, crossover design pilot trial. All subjects were provided a list of foods to avoid during the 7-day run-in period prior to the intervention and throughout the duration of the study. They include *Vaccinium* fruits and high polyphenolic foods including cranberries, blueberries, lingonberries, chocolate, red wine, strawberries, grapes, cherries, plums, blackberries, *etc.* On the morning of the intervention between 7:00 and 10:00 AM, a baseline urine sample (*t* = 0 h) was collected from all subjects after overnight fasting. Participants were then randomized to receive either 1 cranberry chew or 1 placebo chew with 240 mL water. The cranberry chew contained cranberry fruit extract, apple juice concentrate, apple puree and pectin. The placebo chew was made from apple juice concentrate, apple puree and pectin and formulated to match the texture and appearance of the active chew without cranberry. Each type of chew was randomly assigned a 3-digital code generated by a computer system. Both volunteers and investigators were blinded to the treatment. Clean-catch urine samples were collected at 0–3, 3–6, 6–9 and 9–12 h post-consumption. Multiple urine collections made within each time interval were pooled. In the same day between 7:00 and 10:00 PM (*t* = 12 h), participants consumed another 1 cranberry chew or 1 placebo chew with 240 mL water. The following morning, an overnight urine sample (*t* = 12–24 h) was self-collected and submitted to the clinic lab. In the afternoon and evening, urine samples were also collected at *t* = 24–30 h and *t* = 30–36 h. After a one-week wash out, participants then began the alternative regimen and repeated the protocol. All urine samples (0, 0–3, 3–6, 6–9, 9–12, 12–24, 24–30, 30–36 h) were aliquoted and kept in a −80 °C freezer until analysis. All experiments were performed in accordance with the Guidelines: Policy for Human Subjects Protection and the Institutional Review Board. Experiments were approved by the ethics committee at the Office of Research and Regulatory Affairs, Rutgers University. Informed consent was obtained from human participants of this study.

2.2 Mannose-resistant hemagglutination (MRHA) assay (P-type *E. coli*)

Anti-adhesion activity against P-fimbriae *E. coli* was tested using a MRHA assay. The ability of urine samples to suppress agglutination of mannose-resistant human red blood cells (HRBC; A1, Rh+) with a clinical *E. coli* isolate was evaluated according to protocols as previously described.[21,22] Results were reported on an ordinal scale with anti-adhesion activity as follows: 0 = 0% adhesion inhibition; 1 = 50% inhibition; and 2 = 100% inhibition. Negative controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + urine, and HRBC + urine. A well containing bacteria + HRBC served as a positive control for agglutination.

2.3 Type 1 fimbriated *E. coli*-uroepithelial cell anti-adhesion assay

Gram-negative *E. coli* strain UTI89 was originally isolated from a woman with acute cystitis.[26] Anti-adhesion activity against type 1 fimbriated *E. coli* was measured by first incubating

BacLight Green labeled bacteria with urine samples or with myricetin in a 96-well microplate for 30 min at 36 °C. Myricetin was applied as a positive control due to its known bacterial anti-adhesion capability.[15,27,28] In our previous collaborative research, myricetin dose-dependently inhibited *E. coli* adherence with a half-maximal inhibitory concentration (IC_{50}) of 0.88 µg mL^{-1}.[15] Briefly, 200 µL of treated bacteria were transferred to the solid black 96-well microplate containing fixed uroepithelial cells, followed by centrifugation at 500g for 2 min. The plate was incubated at 37 °C under a 5% CO_2 atmosphere for 1 h. To remove the excess unbound bacteria, the supernatant was removed and each well was washed once with DPBS without Ca^{2+}/Mg^{2+}. Fluorescence intensity (FI) was acquired in a microplate reader and quantified with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All urine samples and the positive control compound were tested in triplicate and standardized to creatinine in the urine.

2.4 Type 1 anti-adhesion activity and anti-adhesion potency determination

A standard inhibition curve was generated by incubating BacLight labeled bacteria with myricetin at different concentrations (0, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg mL^{-1}) at 35 °C for 30 min. A linear regression equation was generated based on the fluorescence intensity (FI) of standards. Myricetin equivalency in each sample was projected from the regression equation based on FI in individual samples. Results were expressed as µg myricetin per mL. The final anti-adhesion activity was adjusted by dividing the myricetin equivalency in each urine sample by its respective creatinine content, expressed as µg myricetin per mg of creatinine.

The anti-adhesion potency of individual urine samples was expressed as % of anti-adhesion activity with respect to the IC_{50} of all myricetin standard inhibition curves recorded. The mean IC_{50} of the myricetin standard curve is 1.037.

$$\text{Anti} - \text{adhesion potency} = \frac{\text{anti} - \text{adhesion activity}}{\text{average } IC_{50} \text{ of myricetin standard curve}}$$

2.5 Analytical measurement of bioactive components in chews

The total proanthocyanidin (PAC) content was determined in a 4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay with an absorbance at 640 nm wavelength. The assay utilized either a procyanidin A2 dimer as a reference standard (BL-DMAC method, adapted from the study by Prior *et al.*) or a proprietary reference standard based on an isolated fraction of purified cranberry PAC developed by Ocean Spray Cranberries, Inc. (OSC-DMAC method).[29,30] The standard used in the OSC-DMAC method would represent more accurately of spectrum of degree of polymerization (DP) of PAC in the cranberries, which would expect to yield a higher value of PAC in comparison with the BL-DMAC method. The DP profile was quantified by using an Agilent 1100/1200 HPLC system with fluorescence detection as described previously.[31] The column was a Develosil Diol 100A (250 × 4.6 mm, 5 µm particle size) with a

flow rate of 1 mL min^{-1}. The mobile phase was a binary gradient consisting of acidic acetonitrile (solvent A, acetonitrile : acetic acid, 98 : 2, v : v) and acidic aqueous methanol (solvent B, methanol : water : acetic acid, 95 : 3 : 2, v : v : v). The starting mobile phase condition is 7% B; hold isocratic for 3 min. Subsequently, solvent B was ramped to 37.6% for 57 min and to 100% B for 3 min thereafter. The condition was held at 100% B for 7 min prior to returning to the starting condition. Fluorescence detection was determined with an excitation and emission wavelength of 230 and 321 nm, respectively. Data obtained from the BL-DMAC method should be interpreted as milligrams (mg) of procyanidin A-2 equivalents per gram (g) of powder or millilitre (mL) of juice. The data generated using the Ocean Spray method (OSC-DMAC) to measure PACs should be interpreted as milligrams (mg) of cranberry specific PAC equivalents per gram (g) of powder or millilitre (mL) of juice.

Total phenolics was measured using an improved Folin–Ciocalteu method and absorbance was measured at 765 nm wavelength.[32]

Anthocyanins were analyzed according to Brown and Shipley[33] using a HPLC system (Agilent 1260 Infinity, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). The mobile phase consisted of solvent A (deionized water : 85% phosphoric acid, 99 : 1, v : v) and solvent B (deionized water : acetonitrile : 85% phosphoric acid, 50 : 49 : 1, v : v). The gradient was as follows: 0–1 min, 0–10% B linear; 1–28 min, 10–50% B linear; 28–32 min, 50–75% B linear; and 32–32.1 min, 75–10% B linear; 32.1–35 min, 10% B isocratic. The column temperature was set at 25 °C and data were collected at 520 nm (DAD). Peaks were compared to those of commercially available cyanidin-3-galactoside, cyanidin-3-glucoside, and peonidin-3-glucoside standards (ChromaDex, Irvine, CA USA).

Flavonoid analyses were performed on an Agilent 1260 HPLC system (Agilent 1260 Infinity, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a binary pump, an autosampler and a DAD. Chromatographic separation was carried out with a Phenomenex Kinetex 2.6µ F5, 100 Å 100 × 4.6 mm column (Torrance, CA, USA). The mobile phase consisted of solvent A (deionized water : trifluoroacetic acid, 99.9 : 0.1, v : v) and solvent B (acetonitrile : methanol, 70 : 30, v : v). The gradient was as follows: 0–2 min, 14% B isocratic; 2–9 min, 14–18% B linear; 9–22 min, 18–43% B linear; 22–23 min, 43–90% B linear; 23–24 min 90% B isocratic, and 24–25 min, 90–14% B linear. The column temperature was set at 30 °C and data were collected at 360, 320 and 280 nm (DAD). Peaks were compared to those of commercially available standards.

2.6 Statistical analyses

Non-parametric analyses were conducted in a Minitab 18 (Minitab, Inc., State College, PA, USA). For type-P anti-adhesion assay, the difference in anti-adhesion activity between the cranberry chew and placebo chew at each time point was evaluated by the Wilcoxon signed rank test. Due to the highly skewed dataset in type-1 anti-adhesion assay, the Mann–Whitney test was assigned to determine the significant treatment effect

between the cranberry chew and placebo chew. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Analytical characterization of the cranberry chew

One 9 g serving of the cranberry chew has a caloric content of 25 kcal and 6 g of sugar with levels of important phenolic con-

Table 1 Analytical characterization of cranberry chew

	Cranberry chew	Placebo chew
Serving size (g)	9	9
Calories (kcal)	25	25
Total carbs/sugar (g)	7/6	7/6
Cranberry fruit extract	YES	NO
Vitamin C (mg)	ND	ND
Proanthocyanidins by OSC-DMAC (mg)	120.14 ± 17.27	ND
Proanthocyanidins by BL-DMAC (mg)	29.35 ± 6.08	ND
Total phenolics by Folin–Ciocalteu (mg)	162.09 ± 4.62	14.00 ± 0.85
Total phenolic acid content (mg)	8.47 ± 0.49	ND
Total organic acid content (mg)	530.06 ± 9.07	280.63 ± 79.17
Cyanidin-3-arabinoside (mg)	0.40 ± 0.17	0.01 ± 0.00
Cyanidin-3-galatoside (mg)	0.78 ± 0.35	0.03 ± 0.00
Cyanidin-3-glucoside (mg)	0.03 ± 0.01	ND
Peonidin-3-arabinoside (mg)	0.42 ± 0.18	0.01 ± 0.00
Peonidin-3-galatoside (mg)	0.99 ± 0.46	0.05 ± 0.00
Peonidin-3-glucoside (mg)	0.10 ± 0.04	ND
Total anthocyanin content (mg)	2.72 ± 1.21	0.76 ± 0.03
Myricetin (mg)	0.61 ± 0.11	ND
Myricetin-3-O-galactoside (mg)	2.27 ± 0.20	ND
Myricetin-3-O-rhamnoside (mg)	0.58 ± 0.16	ND
Quercetin (mg)	1.32 ± 0.11	ND
Quercetin-3-O-galactoside (mg)	5.03 ± 0.22	ND
Quercetin-3-O-rhamnoside (mg)	1.17 ± 0.11	ND
Unidentified flavonols (mg)	2.02 ± 0.44	ND
Total flavonol content (mg)	13.00 ± 1.35	ND

Data are expressed as mean ± standard deviation. ND: not detected.

stituents (Table 1) similar to one 8 oz serving of a cranberry beverage tested in the large clinical trial in women with recurrent UTIs.[6] The PAC content in the cranberry chew was 24.39–36.13 mg A2 equivalents per serving using BL-DMAC assay, and 103.37–137.87 mg per serving by the OSC-DMAC method (Table 1), which is higher than that in most other cranberry supplements on the market (unpublished internal data). The degree of polymerization (DP) of cranberry PACs in chew was quantified. Monomers (DP1), dimers (DP2), trimers (DP3), oligomers (DP4–7), and polymers (DP ≥ 10) accounted for 1.6%, 4.2%, 3.8%, 16.3%, and 74%, respectively, of total cranberry PACs (120 mg per 9 g chew by OSC-DMAC method). The characteristic anthocyanin and flavonol profiles of cranberry fruits were detected in cranberry chew as shown in Table 1. The total phenolic content in cranberry chew was 162.1 mg gallic acid equivalents per serving.

3.2 Urinary anti-adhesion activity against P-fimbriated *E. coli*

Urinary anti-adhesion activity against P-type *E. coli* was evaluated using MRHA assay and the results are shown in Fig. 1. No anti-adhesion activity was reported in 0 h baseline urine samples taken prior to the consumption of treatment products. Significantly greater ($p < 0.05$) anti-adhesion activity was observed in urine samples collected from volunteers that consumed cranberry chew compared to placebo chew at all collection time points except for 24–30 h and 30–36 h. The activity peaked between 3 and 6 h following the consumption of the first dose of cranberry chew and lasted up to 12 h. These results are consistent with our previous study in which urinary anti-adhesion activity was significantly greater compared to placebo after consumption of cranberry extract beverage/cranberry extract and juice beverage, with activity peaking between 3 and 6 h, but lasting only 9 h.[22] To build on the learning from the previous study, volunteers were given a second dose cranberry chew to consume at $t = 12$ h, with additional urine collections. Urine collected in the morning after the second
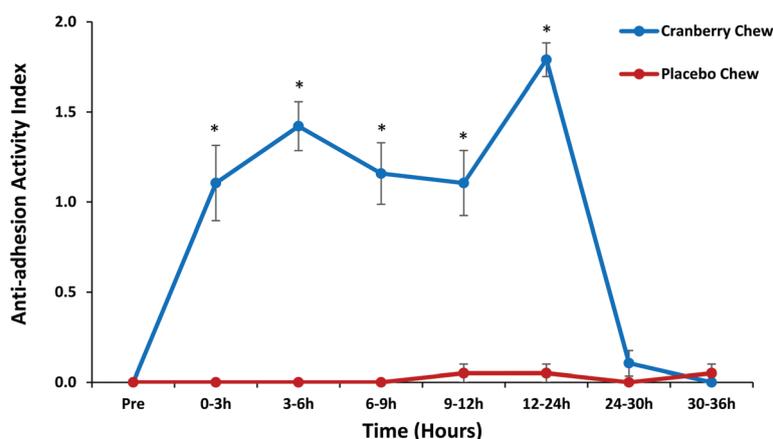

Fig. 1 Urinary anti-adhesion activity against P fimbriated uropathogenic *E. coli* after consumption of two divided doses of cranberry chew. Data are expressed as mean ± SEM ($n = 20$). Anti-adhesion index key: 1 = 50% anti-adhesion activity, 1.5 = 75% anti-adhesion activity and 2.0 = 100% anti-adhesion activity. * indicates that post-consumption anti-adhesion activity of cranberry chew was significantly greater compared to that of placebo chew ($p < 0.05$).

dose showed the highest anti-adhesion activity (AAA = 1.8, Fig. 1) over the time course of the study, indicating the possibility of extending the benefit to the urinary tract from the second dose of cranberry chew. Anti-adhesion activity decreased to near baseline values at 24–30 h and 30–36 h after consumption of the first dose. No anti-adhesion activity was reported for placebo chew at any time.

3.3 Urinary anti-adhesion activity against type 1 *E. coli*

In the present study, we successfully optimized and validated the published fluorometric microplate assay by Kimble *et al.*[15] and demonstrated the urinary anti-adhesion effects on type 1 *E. coli* following cranberry chew consumption. The data (Fig. S1A and S1B†) showed that a BacLight Green concentration of 1000 μM and a ratio of UPEC : uroepithelial cells of 600 : 1 were optimal and physiologically relevant.

Urine samples collected from volunteers who consumed cranberry or placebo chews were analyzed using the current fluorescent-labeled UPEC and uroepithelial cell assay format to evaluate anti-adhesion effects on type 1 *E. coli*. Urine samples collected from four subjects were not tested due to anti-adhesion activity being detected in their pre-study background urine samples. This phenomenon could be due to the production of endogenous adhesion inhibitors such as Tamm–Horsfall glycoprotein.[34,35] Urine samples from a total of 16 subjects were analyzed further for their type 1 anti-adhesion effect (Fig. 2). Urine samples other than those collected at 24–30 h and 30–36 h exhibited some anti-adhesion activity on type 1 *E. coli* following cranberry chew consumption and the results obtained were consistent with results obtained in the MRHA assay using P-fimbriae *E. coli*. The type 1 anti-adhesion activity peaked at 3–6 h ($p = 0.024$) after the first chew dose and lasted up to 9 hours ($p = 0.043$) when the effect was both significantly different from placebo chew. The activity decreased in urine collected from 9–12 h and peaked again in

the 12–24 h ($p = 0.12$) urine samples after consuming a second dose cranberry chew (Fig. 2).

4. Discussion

Cranberries are known to be a rich source of phenolic compounds including polymeric flavan-3-ols (PACs), anthocyanins and flavonols. High levels of phenolic constituents were detected in cranberry chew, with PAC levels (Table 1) greater than many other commercial cranberry supplements (unpublished internal data). The levels of phenolic content were similar to those in a cranberry beverage recently found in a clinical trial to reduce symptomatic UTI by 39%.[6] Cranberry PACs are unique in the structure with a higher percentage of A-type bonds, compared with other commonly consumed fruits. In a previous study conducted by Feliciano and colleagues,[36] cranberry PACs with one A-type bond were found most abundant in DP between 2 and 5. PACs with two A-type bonds were most abundant in DP between 6 and 9. PACs with DP 10 and 11 exhibited mainly three A-type bonds. In another study performed by the same research group,[37] results showed that 94.5% of cranberry PACs contained at least one A-type bond and 26% PACs had at least two A-type bonds. This was in contrast to the DP profile of apple PACs with 88.3% as B-type bonds. Compared with apple PACs, cranberry A-type PACs were significantly more effective in increasing bacteria agglutination and decreasing bacteria invasion to epithelial cells caused by extra-intestinal pathogenic *E. coli*.[37] The result was consistent with a previous finding that the urinary tract benefit was only observed following cranberry intervention but not after the use of B-type PAC rich food, such as grape, apple juice, green tea, and dark chocolate.[13] It is noted that oligomers and polymers of PACs are flavan-3-ol with a high molecular weight and were minimally absorbed in the upper GI tract[38] until they reached
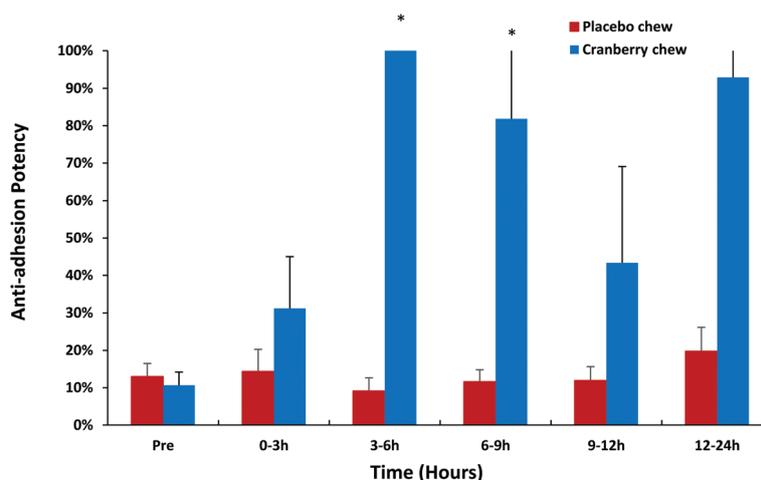

Fig. 2 Urinary anti-adhesion activity against type 1 fimbriated uropathogenic *E. coli* after consumption of two divided doses of cranberry chew. Data are expressed as mean ± SEM ($n = 16$). The anti-adhesion potency of individual urine samples was expressed as % of anti-adhesion activity with respect to the mean half-maximal inhibitory concentration (EC_{50}) of all myricetin standard curves recorded. The mean EC_{50} of the myricetin standard curve is 1.037. * indicates that post-consumption anti-adhesion activity of cranberry chew was greater compared to that of placebo chew ($p < 0.05$).

colon for fermentation. It is likely that the bacterial metabolites from PACs that were absorbed and excreted in the urine elicit the benefit. Ou *et al.* showed that cranberry procyanidins did not yield the exclusive metabolite profile after gut bacteria fermentation, compared with apple procyanidins but had a slower degradation rate by gut bacteria, yielding higher levels of certain bacteria-derived metabolites compared to the apple procyanidins.[39] This suggested that bacteria derived enzyme(s) might interact and utilize A-type and B-type PACs differently due to the difference in the configuration of A-type and B-type PACs.

Anthocyanins make up another important class of polyphenols in cranberries, providing the red color of the berries and eliciting certain bioactivities, such as antioxidant and anti-inflammatory activities.[40] The levels were equivalent to those found in raw cranberry fruits, suggesting that most of the anthocyanins are retained in the cranberry chew following the manufacturing process. It has been reported that more than 20 different glycosides of flavonols[41–43] are found in cranberries and the contents are nearly double those found in commonly consumed fruits including pomegranates and grapes.[44,45] The most abundant flavonols in cranberries are quercetin-3-galactoside and myricetin-3-O-galactoside which were retained in the cranberry chew at levels similar to fresh cranberries (29.68 mg quercetin-3-galactoside 14.8 mg myricetin-3-O-galactoside per 100 g fruit) (Table 1). Quercetin and quercetin-3-rhamnoside (Table 1) were much higher compared to those in cranberry fruits (0.67 mg quercetin and 2.65 mg quercetin-3-rhamnoside per 100 g fruit).[9] The cranberry chew also contained a high level of various phenolic acids, such as benzoic acid, chlorogenic acid, 3,4-hydroxylbenzoic acid, and *p*-coumaric acid (Table 1), which may possess high anti-microbial properties.[28]

In vitro studies have shown cranberries and certain polyphenolic compounds to possess some anti-adhesion activity when directly incubated with bacteria and cultured cells.[13–18] However, it is difficult to determine if these compounds will be active *in vivo* and at what concentrations. Rodríguez-Pérez *et al.*[17] reported that phenolics including dihydroferulic acid glucuronide, procyanidin A dimer, quercetin glucoside, myricetin and prodelphinidin B decreased the surface hydrophobicity of UPEC *in vitro*, resulting in potential reductions in adhesive forces. By using the atomic force microscopy technique, Gupta and colleagues[16] found that a crude cranberry extract as well as flavonols including quercetin-3-O-galactoside and myricetin-3-O-galactoside showed anti-adhesion properties on both non-pathogenic and antibiotic resistant *E. coli* strains. In another study performed by Rafsanjany *et al.*,[25] adhesion of *E. coli* strain NU14, which expresses type 1 fimbriae, was reduced by both the cranberry extract and the PAC-free cranberry extract, which is consistent with previous studies demonstrating that PACs inhibit P-type *E. coli*.

Although it has been reported that certain cranberry parent compounds are bioavailable, the concentration in urine is rather low.[46] Recent studies tested gut microbial-derived cranberry metabolites and their potential bacteria anti-adhesion activity. Two of the most abundant metabolites produced following cranberry consumption, hippuric acid and α-hippuric

acid, exhibited *in vitro* anti-adhesion effects on UPEC at physiologically relevant concentrations (100, 250 and 500 μM). In addition, catechol, benzoic acid, vanillic acid, phenylacetic acid and 3,4-dihydroxyphenylacetic acid were able to inhibit the adherence of UPEC to uroepithelia cells in a concentration-dependent manner from 100–500 μM,[18] although further studies are needed. One of the cranberry PAC degradation products, valerolactone and its derivatives, showed anti-adhesion properties at a concentration of 100 μM, indicating their potential biologically relevant activity in urine.[19]

Despite encouraging observation from cell-based studies, few studies have investigated the *ex vivo* urinary anti-adhesion effect against type 1 *E. coli*, combining both a human intervention component and an *in vitro* component to study exposure to the cranberry product occurring at expected intake levels in humans and the presence of anti-adhesive cranberry components in the urine. Di Martino *et al.*[24] found that urine from cranberry juice consumers inhibited the adhesion of *E. coli* with type 1 fimbriae. In another study by Rafsanjany *et al.*,[25] subjects consumed 600 mg cranberry extracts for either 3 or 7 days. Urine collected from those participants had a significantly enhanced anti-adhesion effect on UPEC strain NU14, a clinical cystitis isolate, indicating a beneficial effect of cranberry consumption on type 1 *E. coli*-associated UTIs.

The current study demonstrates *ex vivo* urinary anti-adhesion activity against both P-type and type 1 *E. coli*, following consumption of cranberry formulated in a chew product. Interestingly, a sustained anti-adhesion benefit was achieved for up to 24 hours when two cranberry chews were consumed daily, one in the morning and one in the evening. Our MRHA results demonstrating that consumption of the cranberry chew supplement helped to prevent P-type *E. coli* from adhering to cell receptors are consistent with other reports using cranberry juices, dried cranberries, powders, and capsules as test products.[9,13,21,47,48]

5. Conclusions

In conclusion, our study demonstrated that human urine following consumption of cranberry +health™ cranberry supplement (cranberry chew) had the ability to inhibit the adherence of both P type and type 1 uropathogenic *E. coli*. Results utilizing assays specific for each bacterial type indicate that consuming a second dose of cranberry chew 12 hours after the initial dose provided extended protection from bacterial adhesion for up to 24 hours. Further human trials are needed to correlate the level of *ex vivo* anti-adhesion activity with prevention of clinical UTIs.

Conflicts of interest

Haiyan Liu, Derek J. Zhang and Christina Khoo are employees of Ocean Spray. Dr Amy B. Howell is an independent university researcher and collaborator with no conflict of interest to declare.

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