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ARTICLE

Differences in Urinary Bacterial Anti-Adhesion Activity after Intake of Cranberry Dietary Supplements with Soluble versus Insoluble Proanthocyanidins

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ABSTRACT

A number of clinical trials support the use of standardized cranberry supplement products for prevention of urinary tract infections; however, products that are not well-characterized for sufficient levels of bioactive components may contribute to negative clinical outcomes. Cranberry supplements for consumer use are not regulated and can be formulated different ways using cranberry juice, pomace or various combinations. This can lead to consumer confusion regarding effectiveness of individual products. The current study compared two commercial supplement products, one made from cranberry juice extract and the other from a blend of whole cranberry. The influence of formulation and proanthocyanidin (PAC) solubility on in vitro and ex vivo P-fimbriated *Escherichia coli* bacterial anti-adhesion activity (AAA) was determined. Both supplement products as well as whole, frozen cranberries were chromatographically separated into crude polyphenolic, sugar and acid fractions. In vitro AAA testing of all fractions confirmed that only those containing soluble PACs elicited activity. The cranberry juice extract product had higher soluble PAC content than the whole cranberry blended product, which contained mainly insoluble PACs. The influence of soluble and insoluble PAC levels in each product on the urinary (ex vivo) AAA was determined following ingestion. The juice extract product was associated with significantly higher urinary AAA than that of the whole berry blended product when consumed once daily over the 1-week intervention period.

KEYWORDS

bacterial anti-adhesion; cranberry; dietary supplements; proanthocyanidins; urinary tract

Introduction

Cranberry (*Vaccinium macrocarpon* Ait.) is widely utilized by consumers for maintenance of urinary tract health. Recent systematic reviews and meta-analyses support the use of cranberry for reducing risk of urinary tract infection (UTI), especially in women and children (Wang et al. 2012; Micali et al. 2014; Fu et al. 2017; Huang et al. 2017;

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Luís et al. 2017; Roshdibonab et al. 2017). However, previous inconsistent clinical results may be due to use of non-standardized investigational products with sub-efficacious doses, and variability within product formulations across different categories (juice, dried powder supplements, dried whole fruit), making conclusions and comparisons to other intervention trials difficult (Jepson et al. 2012; Howell 2013).

Consumer use of cranberry dietary supplements is on the rise with sales in the United States of nearly $90,000,000 in 2019 (Smith et al. 2020). Cranberry supplements are typically made from different components of the fruit and can be composed of either whole dried cranberry, dried juice extracts, dried pomace remaining after the juicing process or various combinations of these. Product manufacturers are not required by the U.S. Food and Drug Administration to list the cranberry components used in formulation. Previous studies have found substantial variability in the composition and bioactivity of cranberry supplements used by consumers to maintain urinary tract health (Krenn et al. 2007; Sánchez-Patán et al. 2012; Chughtai et al. 2016; Mannino et al. 2020), indicating that research is needed to determine which raw cranberry ingredients are contributing to the benefits. Accurate determination of the efficacy of these different supplement formulations can be challenging and involves utilizing appropriate methods to accurately quantify active compounds and test their bioactivity.

One bioassay is based on an important mechanism of action of cranberry, the prevention of bacterial adhesion to uroepithelial cells (Sobota 1984; Zafriri et al. 1989; Ofek et al. 1991), which is the initial step in the urinary infection process (Beachey 1981). Uropathogenic *Escherichia coli* (*E. coli*) bacteria cause the majority of UTIs. They produce adhesin colonization factors on their surfaces called fimbriae (pili) which are hair-like filamentous structures. Adhesins on the fimbrial tips utilize their capacity as lectins (sugar-binding proteins) for attachment of bacteria to cell surface sugar receptors on uroepithelial cells (Roberts et al. 1994). The majority of the adhesins are either PapG mannose resistant (MR) on P-fimbriae, or FimH mannose-sensitive (MS) on Type 1 fimbriae (Johnson et al. 1998; Kaper et al. 2004). *E. coli* expressing P-fimbriation are associated with bladder and kidney infections, especially pyelonephritis, which can cause serious symptoms (Roberts et al. 1994). The ability of cranberry to prevent bacterial adhesion to uroepithelial cells can be measured directly by counting adhered bacteria. But, since bladder cells have receptors for multiple adhesins, the assays are not specific and cannot attribute the inhibitory activity to a particular *E. coli* fimbrial type (Neter 1956; Zafriri et al. 1989). Validated microscopy-based hemagglutination assays able to detect subtle differences in bacterial lectin binding specificity to cellular surfaces (Vagarali et al. 2008; Mrázková et al. 2019) have been used to determine inhibition of specific agglutination by foods and isolated compound fractions (Toivanen et al. 2010; Eltigani et al. 2019). The MR hemagglutination (MRHA) bioassay is routinely used to measure the P-fimbriated *E. coli* bacterial anti-adhesion activity (AAA) of either whole cranberry products (Zafriri et al. 1989; Ofek et al. 1991; Howell et al. 1998; Foo et al. 2000a, 2000b; Gupta et al. 2007, Chughtai et al. 2016) or urine collected following consumption of the products (Howell et al. 2005; Valentova et al. 2007; Howell et al. 2010; Howell et al. 2015; Kaspar et al. 2015; Liu et al. 2019; Singh et al. 2016). Measuring the in vitro AAA of whole products is used to determine potency prior to ingestion (Gupta et al. 2007; Chughtai et al. 2016) and for monitoring shelf-life. Ex vivo urinary AAA

helps determine dose-response and persistence over time (Valentova et al. 2007; Howell et al. 2010; Kaspar et al. 2015). Urinary assays more closely mimic conditions in vivo and provide additional biological relevance in that urine collected following cranberry intake is used as the substrate for incubating uropathogenic bacteria at levels indicative of clinical UTI.

The majority of AAA has been linked to polyphenolic compounds in cranberry, especially A-type proanthocyanidins (PACs) found in the water-soluble juice portion of cranberry fruit (Howell et al. 1998; Foo et al. 2000a, 2000b; Gupta et al. 2007; Gupta et al. 2012) and their metabolites (de Llano et al. 2015; Mena et al. 2017). The insoluble, non-extractable PACs complexed with cellulose fibers in the cranberry skins that remain after the juicing process (Roopchand et al. 2013; Gullickson et al. 2020) have not, to date, been shown to affect bacterial adhesion. Urinary AAA has been positively correlated with soluble PAC content in a dose-dependent manner in juice-derived cranberry supplements (Lavigne et al. 2008; Howell et al. 2010). Soluble cranberry PACs inhibit primarily P-fimbriated *E. coli* adhesion with the unusual double A-type linkages in the molecules being potentially important structural features in the anti-adhesion process (Foo et al. 2000a, 2000b). A few studies demonstrate that adhesion of Type 1 (MS) *E. coli* can also be inhibited to a certain extent by cranberry (Rafsanjany et al. 2015; Liu et al. 2019) and the A-type PACs (Lavigne et al. 2008). Specific flavonols (Scharf et al. 2020) and oligosaccharides found in cranberries (Hotchkiss et al. 2015) may also inhibit Type 1 *E. coli* adhesion. However, more research is warranted to determine their biological relevance.

Precise measurement of PAC content in cranberry supplements is important to ensure correct consumer product labeling, efficacy monitoring, shelf-life determination and formulation of standardized materials for research studies. Due to the complexities of the PAC structures and A-type PAC linkages, results obtained from most PAC quantitation methods are often erroneous and may not be reproducible (Mole and Waterman 1987; Krenn et al. 2007). Several analytical procedures, such as Bate-Smith and European Association for the Valorization of Cranberry (EuraCran) overestimate PAC content because the anthocyanin fraction, which is not removed prior to spectral analysis of the anthocyanidins formed from the hydrolysis and butanol extraction of PACs, is added in as part of the total PAC measurement (Waterhouse et al. 2000). The aldehyde condensation of 4-(dimethylamino)cinnamaldehyde (DMAC), a colorimetric method widely used in the cranberry industry is more accurate than other colorimetric methods, inexpensive, rapid, simple to perform and less likely to have interferences from other sample components (Cunningham et al. 2002).

Due to the heterogeneous mixture of juice and pomace ingredients in many cranberry dietary supplements, different methods with specific reference standards need to be utilized to accurately quantify both soluble and insoluble PACs individually. Soluble PAC levels in juice-derived products are routinely measured using DMAC (Prior et al. 2010; Krueger et al. 2013; Sintara et al. 2018; Birmingham et al. 2020) (www.dmac-assoc.org). The 36 mg PAC level often reported in cranberry products refers specifically to the soluble PAC fraction measured exclusively with the procyanidin A2 reference standard (DMAC/A2). It is the recommended daily intake of soluble PAC in products according to large meta-analyses on cranberry clinical trials for UTI prevention (Jepson et al.

2012; Jepson 2013). It demonstrated positive effects in human intervention trials for reduction in UTI measures (pyuria and bacteriuria), using a 300-mL serving of cranberry juice drink (Avorn et al. 1994). Additional studies have demonstrated significant urinary AAA (Lavigne et al. 2008; Howell et al. 2010, 2015) and reductions in recurrent UTI (Uberos et al. 2012; Maki et al. 2016; Occhipinti et al. 2016; Ledda et al. 2017; Thomas et al. 2017) when cranberry products with 36 mg of soluble PAC were consumed. Insoluble PACs in whole berry or pomace-derived cranberry supplements can be measured accurately using butanol-HCl (BuOH-HCl) with the c-PAC reference standard (Gullickson et al. 2020).

Given the inherent variability among cranberry supplements and consumer interest in purchasing efficacious products for maintenance of urinary tract health, more clarity is needed with respect to bioactivity of products made from different components of the fruit. Therefore, this study was undertaken utilizing two commercial cranberry dietary supplements, one derived from cranberry juice and the other from the whole berry, to determine: (1) how the relative levels of soluble and insoluble PAC in each product influence the AAA of the whole products against P-fimbriated *E. coli*, (2) the differences in AAA among isolated cranberry fruit and supplement fractions containing distinct phytochemical compositions to elucidate which are bioactive, (3) the differences in daily ex vivo urinary bacterial AAA between the two supplement products when administered to two groups of participants, and any cumulative effects on urinary AAA after a one-week period.

Materials and methods

Cranberry supplement investigational product information

Two commercially available over-the-counter dried cranberry dietary supplement products sold in the United States were purchased online. Product 1: AZO Cranberry caplets, recommended serving size of 2 caplets (800 mg/caplet) containing a total of 500 mg Pacran, 60 mg vitamin C, 110 mg Calcium, 30 mg *Bacillus coagulans* and other ingredients (excipients and coloring agents). Pacran, as stated on the packaging, is derived from whole cranberry fruit. PAC content is not specified. Product one will be referred to as whole cranberry fruit-derived (WCFD). Product 2: ellura capsules, recommended serving of 1 capsule (265 mg/capsule) containing 200 mg Gikacran cranberry juice dry extract with 36 mg PAC and other ingredients (excipients). Packaging states that Gikacran is derived from cranberry juice. Product two will be referred to as cranberry juice-derived (CJD).

Quantification of soluble and insoluble PACs in cranberry supplement products

Quantification of soluble and insoluble PACs in both cranberry supplement products was performed by CPS (Complete Phytochemical Solutions, LLC, Cambridge, WI), and additional soluble PAC testing was performed by ICT (International Chemistry Testing, Milford, MA). Soluble PAC levels were determined utilizing the DMAC method with procyanidin A2 reference standard (DMAC/A2). A butanol-hydrochloric acid (BuOH–HCl) method with a proprietary c-PAC reference standard (not commercially

available) was used for determining insoluble PAC levels (Feliciano et al. 2012; Birmingham et al. 2020).

Isolation of crude extract fractions from frozen cranberries and cranberry supplement products to determine fractions with AAA

A solid-phase chromatography method (Howell et al. 2005) was used to target and isolate crude fractions of PACs, sugars, acids, total polyphenolics, anthocyanins and flavonol glycosides from frozen cranberry fruit (*Vaccinium macrocarpon* Ait. cv. Early Black), which were subsequently tested to determine which fractions have AAA and identify the compound classes responsible for the activity. The same chromatography method was then used for isolating, identifying and AAA-testing the same crude fractions from the WCFD and CJD cranberry supplements, which vary in composition and contain a subset of compounds found in whole cranberry fruit.

Briefly, the cranberry fruit was blended in distilled water (dH_2O) and filtered through cheesecloth. Aliquots of each supplement were pulverized separately and suspended in dH_2O. Each slurry was applied to separate C18 SepPak cartridges (Waters Corp., Milford, MA) that were preconditioned with methanol (MeOH) followed by dH_2O. The sugar fraction was collected as each cartridge was washed with dH_2O then dH_2O:MeOH (85:15) (v/v). The acid fraction was eluted with acidified aqueous methanol. The polyphenolic fraction containing anthocyanins, flavonol glycosides and PACs (confirmed using reverse phase high performance liquid chromatography [HPLC] with diode array detection) was eluted from each cartridge with 1% acetic acid (HOAc) in MeOH (v/v). All fractions were dried under reduced pressure to remove solvent. The polyphenolic fractions were then suspended in 50% ethanol (EtOH), applied to Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO) columns that were pre-equilibrated overnight in EtOH:dH_2O (50:50) (v/v). Anthocyanins and flavonol glycosides were eluted with 50% EtOH and dried to remove solvent and suspended in ethyl acetate (EtOAc):dH_2O (50:50) in a separatory funnel to let two phases form. The flavonol glycosides were eluted with the EtOAc phase and the anthocyanins with the water phase and both fractions dried under reduced pressure. PACs adsorbed to the LH-20 columns were eluted with 80% aqueous acetone and monitored using diode array detection at 280 nm. Acetone was removed under reduced pressure and the resulting purified PAC extracts were dried.

The purity of each crude fraction from cranberry fruit was assessed by Complete Phytochemical Solutions, LLC, Cambridge, WI. The 80% acetone fraction (which targets PACs) and 50% ethanol fraction (which targets anthocyanins and flavonols) collected from the Sephadex LH-20 column were assessed for purity using HPLC with an Agilent Zorbax SB-C18 (Agilent, Santa Clara, CA) column (4.6 × 150 mm). Mobile phase A: 5% acetonitrile (ACN) in water + 0.3% trifluoracetic acid (TFA), and mobile phase B: 0.3% TFA in ACN with a column temperature of 30 °C. Injection volume was 20 µL with a flow rate of 1 mL/min. HPLC chromatograms generated at a wavelength of 280 nm were used to determine if the sugar and acid fractions were free of PACs. DMAC/A2 was used to determine levels of residual soluble PAC in all cranberry fruit fractions which could potentially influence AAA.

Bacterial anti-adhesion assays (in vitro)

Escherichia coli *strain utilized in AAA bioassays*

Uropathogenic *E. coli* wild type bacterial strain isolated from the urine of a human pyelonephritis patient was obtained from Scripps Medical Laboratory, Scripps Clinic, La Jolla, CA. Bacteria were maintained at Rutgers University Marucci Center in a biosafety Level two containment laboratory. Strain was subcultured in tryptose broth at 37 °C for 16 h, streaked on colonization factor antigen (CFA) agar plates and grown overnight at 37 °C to enhance production of P fimbriae. Cultures were retained on agar slants at 4 °C for short-term use over several months and kept frozen at −70 °C in tryptose broth (30% glycerol) in cryogenic vials for long-term storage.

Bacterial AAA of cranberry supplement products (in vitro)

Investigational cranberry supplement products were tested for in vitro bacterial AAA on a per weight basis using the MRHA assay specific for uropathogenic P-fimbriated *E. coli* according to Foo et al. (2000a). WCFD caplet, pulverized with a mortar and pestle and CJD capsule contents were separately suspended (60 mg/mL) in phosphate buffered saline solution (PBS), neutralized to pH 7 with 1 N NaOH and diluted in a twofold dilution series in PBS. P-type *E. coli* bacteria were harvested from agar slants and suspended directly in PBS at pH 7.0 at a concentration of 5×10^8 bacteria/mL of PBS for AAA testing. A 30-μL drop of each dilution in the series was incubated with 10 μL of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn A type Rh + human red blood cells (HRBC) were suspended (3%) in PBS and 10 μL added to each test suspension. Suspensions were incubated for 20 min on a rotary shaker at room temperature, and then, evaluated microscopically for hemagglutination. The dilution concentration at which hemagglutination activity was suppressed by 50% was recorded as the endpoint for the assay and was considered the minimum inhibitory concentration (MIC). The lower the MIC, the higher the AAA of the sample. Anti-adhesion assays were repeated four times on duplicate product samples and the results averaged. Negative controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + test material, HRBC + test material. Positive control well was bacteria + HRBC.

Bacterial AAA of isolated fractions from cranberry fruit and supplement products (in vitro)

Dried crude fractions obtained from cranberry fruit and supplement products were re-suspended in PBS and tested for P-fimbriated *E. coli* AAA in a twofold dilution series, as described in the section above, with a starting concentration of 5 mg/mL. The MIC dilution of each crude fraction was observed under the microscope and photographed to display extent of hemagglutination.

Urinary bacterial AAA following consumption of cranberry supplement products (ex vivo)

The ex vivo study protocol was approved by the Institutional Review Board at Rutgers, The State University of New Jersey. Informed consent was obtained from volunteers prior

to enrollment in the study. As a requirement of study participation, volunteers had to have urines, collected daily for a 2-week period, test negative for production of endogenous bacterial adhesion inhibitors. If any urines were positive for P-fimbriated AAA, those volunteers were excluded from the study. Participants included twenty healthy females and males between the ages of 25 and 60 with no history of current or recurrent UTIs, urinary disorders, diabetes, or antibiotic use within the last six months. Participants refrained from consuming cranberry, lingonberry, blueberry, pomegranate, grape, chocolate, red wine and other high-polyphenolic foods for a 5-day wash out period prior to consuming investigational products and throughout the testing period. Fluid consumption was standardized to 240 mL every 3 h to avoid dilution of urine samples and allow for detection of AAA, if present. Baseline clean-catch urine samples were self-collected by all 20 participants at 7:45 am on day 1, prior to consuming the cranberry products.

Treatment consisted of either two 800-mg WCFD caplets (taken together) for a total intake of 500 mg Pacran (PAC content not disclosed on packaging), or one 265-mg CJD capsule containing 200 mg Gikacran. Products were administered to all 20 participants in a repeated measures design, with a wash-out period of 5 days between each treatment. Participants took the first dose on day 1 at 8 am (time 0) with 240 mL of water, and then, subsequent doses every 24 h for 7 days. Each day over the 1-week period, participants self-collected clean-catch urine at three time points: 0 h (product administration), 8 h and 24 h after product intake. The level at 24 h is also the level at 0 h for the following day. A total of 30 urine samples over each 168-h intervention period were collected from each participant after completing both product intake regimes. Following collection, all urines were frozen by participants and brought to the laboratory at Rutgers where they were kept frozen at $-20\,^{\circ}C$ until analyzed.

For analysis, frozen urines were thawed, centrifuged, filtered (0.45 μm filter) and tested full-strength for bacterial AAA utilizing the MRHA assay specific for uropathogenic P-fimbriated *E. coli* according to Foo et al. (2000a). A 30-μL drop of each urine was incubated with 10 μL of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn HRBCs (A+) were suspended (3%) in PBS and added separately (10-μL drops) to test suspensions, which were then incubated for 20 min on a rotary shaker at room temperature and evaluated microscopically for the ability to prevent agglutination. AAA of each urine sample was scored visually based on a quantitative estimation of percent agglutination of each sample using the following scale: 0 = no AAA, 1 = 50% AAA (moderate activity in the urine), 2 = 100% AAA (high activity in the urine). This scale can be readily converted into approximate percentage of possible AAA by multiplying each raw figure by 50. Anti-adhesion assays were repeated four times per sample and the median was retained. Controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + test material, HRBC + test material and bacteria + HRBC.

Daily AAA values were plotted as adjusted Area Under the Curve (AUC) which allows for the assessment of cumulative dosing effects on AAA for each treatment product. AUC summarizes the effect of the investigational products by integrating the amount under the level curve over time. For AAA, AUC (Percent/Urine Vol $\times$ Day) was calculated by the triangular method. The maximal value of AAA AUC was then standardized as 100.

Table 1. Quantitation of soluble and insoluble proanthocyanidins (PACs) per serving of each commercial cranberry supplement product and associated P-fimbriated *E. coli* bacterial anti-adhesion activity (AAA).

Investigational product	Serving size	Cranberry ingredient	Fruit extract in cranberry ingredient	Soluble PAC[a] A2 standard (mg/serving)	Insoluble PAC[b] c-PAC standard (mg/serving)	AAA whole product (mg/mL)
WCFD (Azo Cranberry)[c]	2 caplets (1600 mg)	Pacran 500 mg	Whole fruit blend	0.94	19.68	ND[d]
CJD (ellura)[e]	1 capsule (265 mg)	Gikacran 200 mg	Juice	35.98	ND	0.23

[a]Measured by DMAC (4-(dimethylamino)cinnamaldehyde) method.
[b]Measured by BuOH-HCl (butanol-HCl) method.
[c](WCFD) Lot # 19024ADM15, Expiry: 10/2020, UPC: 787651420677.
[d]Nondetectable.
[e](CJD) Lot # UG1810, Expiry: 8/2021, UPC: 855859003906.

Statistical analyses

A mixed model for repeated measures was applied to the AUC values. This model includes two fixed factors: Treatment (with two levels: CJD (one capsule) and WCFD (two caplets) and Day (with seven levels), plus the interaction term: Treatment × Day. The results were calculated using a Restricted Maximum Likelihood paradigm. Some variance – covariance matrices did not allow for convergence. For those converging, the Akaike Information Criterion was used to select the best model and led to the selection of a diagonal covariance matrix. A bilateral p of .05 was considered as the significance threshold. All calculations were done using NCSS 2019 (NCSS LLC, Kaysville, UT).

Results

PAC levels and bacterial AAA of cranberry supplement products

Quantification of soluble and insoluble PACs

Soluble PAC content measured by DMAC/A2 was significantly higher in CJD (35.98 mg per 1-capsule serving) than WCFD (0.94 mg per 2-caplet serving; Table 1). Percent soluble PAC was 1.9% in WCFD in the 500-mg Pacran cranberry powder ingredient and 17.9% in CJD in the Gikacran cranberry powder ingredient. Insoluble PAC content measured by BuOH–HCl with c-PAC reference standard was 19.68 mg per 2-caplet serving of WCFD (3.9% of the Pacran powder) and non-detectable (0% of Gikacran) in the 1-capsule serving of CJD (Table 1).

Bacterial AAA (in vitro)

P-fimbriated *E. coli* AAA of WCFD was non-detectable at concentrations of up to 60 mg/mL. AAA of CJD was detectable at a MIC of 0.23 mg/mL (Table 1).

Isolation and determination of crude cranberry fruit and supplement fractions with bacterial AAA

The HPLC chromatograms of the Sephadex LH-20 cranberry fractions show that the 80% acetone fraction targeting PACs was highly purified, with the majority of the

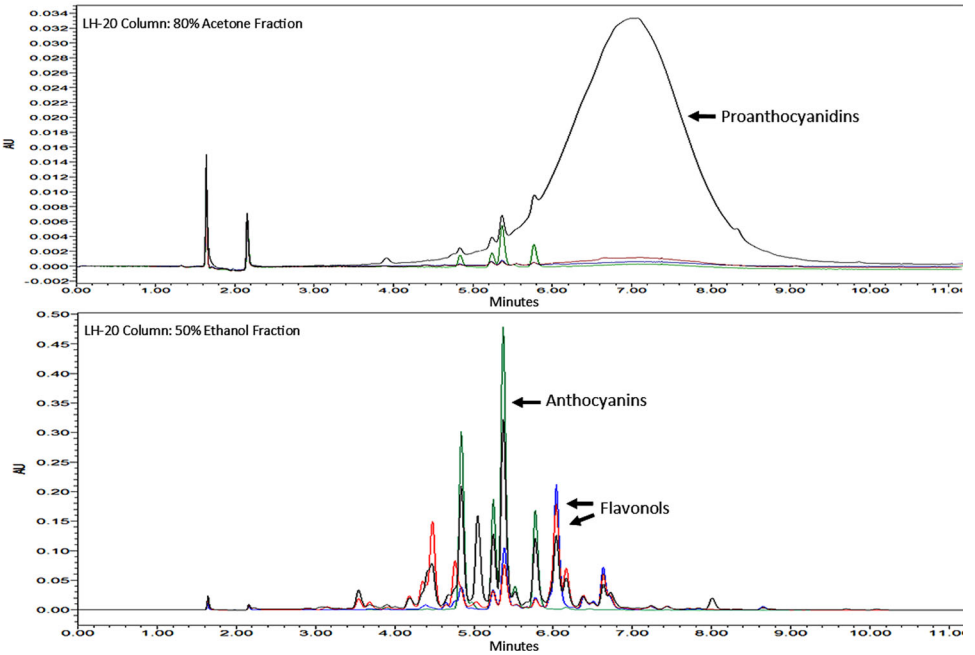


Figure 1. HPLC of Sephadex LH-20 crude cranberry fruit 80% acetone and 50% ethanol fractions collected during chromatographic separation to determine purity.

anthocyanins and flavonols remaining in the 50% ethanol fraction (Figure 1). Comparison of the retention times among the PAC, sugar and acid HPLC chromatograms at 280 nm confirmed that the sugar fraction was virtually free of PACs with very low residual PACs in the acid fraction (Figure 2). Further purity assessment utilizing DMAC/A2 determined that there were very low levels of residual soluble PAC in the acid and anthocyanin/flavonol fractions and no detectable PACs in the sugar fraction (Table 2).

Bacterial AAA was only detectable in the isolated PAC fractions from cranberry fruit and supplement products, which exhibited no hemagglutination in the assay (Figure 3). The PACs isolated from both cranberry fruit and CJD had an AAA MIC of 0.01 mg/mL, while PACs from WCFD had a AAA MIC of 0.15 mg/mL. AAA was non-detectable in crude fractions of sugar, acids, flavonol glycosides and anthocyanins, which displayed 100% hemagglutination (Figure 3).

Urinary bacterial AAA following consumption of cranberry supplement products (ex vivo)

The treatment factor, which summarizes the AUC measured daily throughout one week of treatment for WCFD (mean AUC: 23) and CJD (mean AUC: 94) was highly statistically significant ($p < .00001$; Tables 3 and 4 and Figure 4). The least − square adjusted AUC was standardized so that the highest AAA AUC on one day of the study period is 100, ranges from 87 to 100 for CJD and from 15 to 32 for WCFD (with a standard error of the mean of 2.5). The day factor and the day × treatment interaction term were not significant ($p = .39$ and .94, respectively; Table 3).

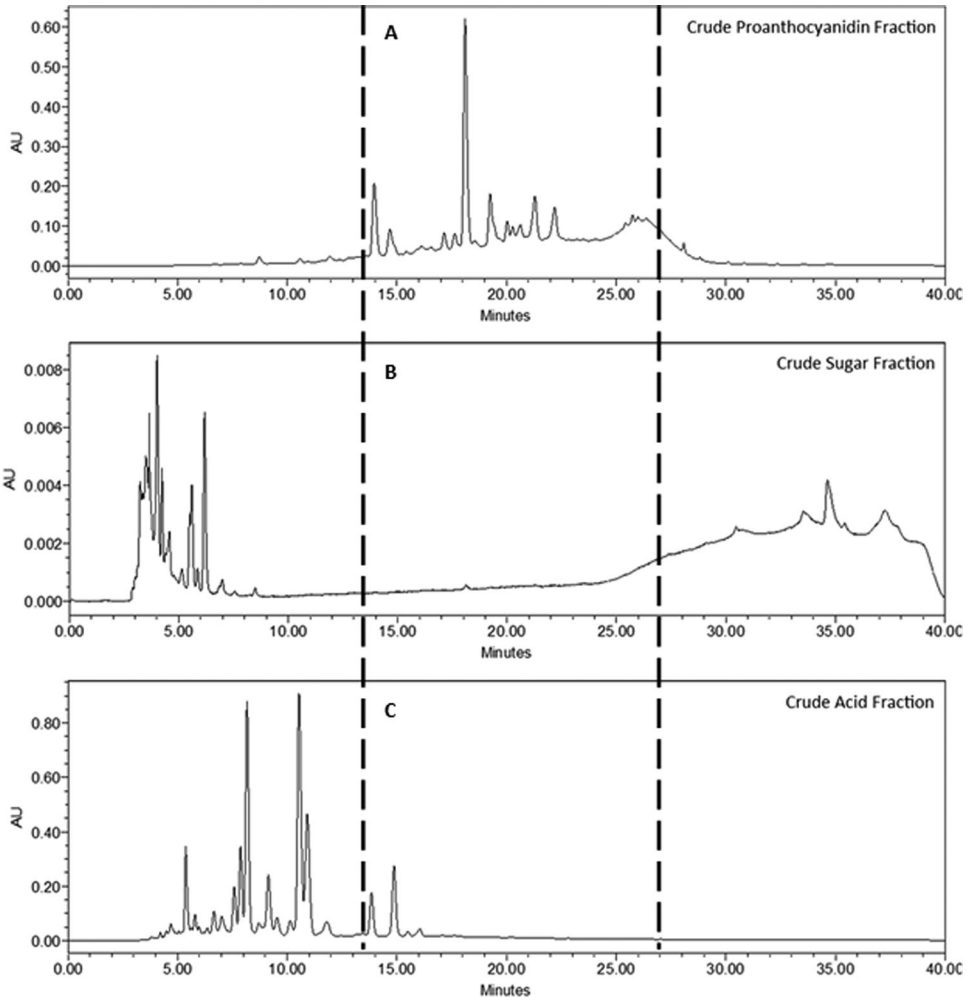


Figure 2. HPLC chromatograms of the crude cranberry proanthocyanidin (PAC), sugar and acid fractions measured at an absorbance of 280 nm to determine purity. A: PAC peaks, B: No PAC peaks, C: Negligible PAC peaks.

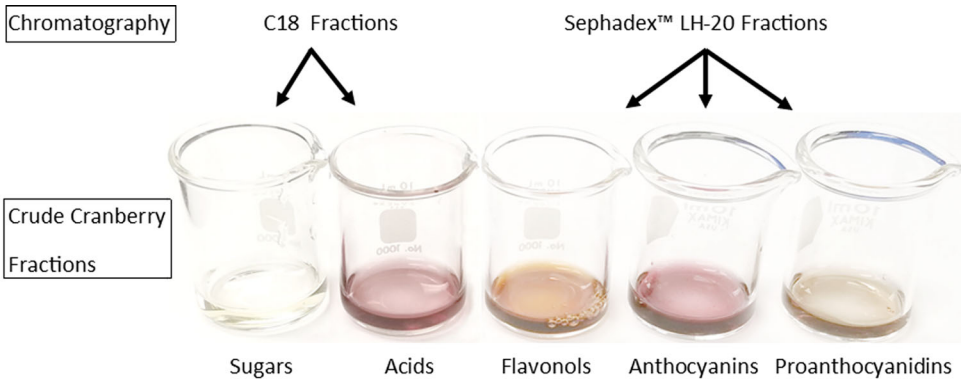
Table 2. Quantitation of soluble proanthocyanidin (PAC) levels in crude cranberry fractions by DMAC[a] method.

Crude cranberry fraction	Soluble PAC (A2 standard)
Anthocyanins/Flavonols	24.5 mg/g
Proanthocyanidins	273 mg/g
Sugars	Below LOQ[b]
Acids	22.6 mg/mL

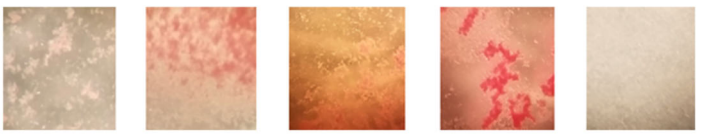
[a]4-(dimethylamino)cinnamaldehyde method using the procyanidin A2 reference standard.
[b]Limit of quantitation.

Discussion

There is substantial clinical evidence to support the use of cranberry for prevention of recurrent UTI. With antibiotic resistance on the rise, it is important to identify strategies for preventing infections so as not to promote resistance development in bacterial



AAA* (mg/mL)	Sugars	Acids	Flavonols	Anthocyanins	Proanthocyanidins
Cranberry Fruit:	ND**	ND	ND	ND	0.01
CJD (ellura®):	ND	ND	ND	ND	0.01
WCFD (AZO®):	ND	ND	ND	ND	0.15



Hemagglutination***	+	+	+	+	-

*Anti-adhesion activity with a starting concentration of 5 mg/mL in a 2-fold dilution series.
**Non-detectable.
***Positive agglutination in photos indicates that test fraction did not elicit AAA. Negative agglutination in photos indicates that test fraction elicits AAA.

Figure 3. Chromatographic separation of cranberry fruit and supplement products into crude fractions and associated P-fimbriated *E. coli* bacterial AAA as demonstrated by the hemagglutination response.

Table 3. Mixed model analysis of variance of daily levels of the efficacy parameter in participants.

Model Term	*F*—value testing Type – III	Num DF	Denom DF	Prob Level
Treatment	399.71	1	259	0
Day	1.07	6	114	0.39
Treatment*Day	0.3	6	114	0.94

strains (Anger et al. 2019). The bacterial AAA of urine following cranberry consumption interrupts the initial step in the infection process. Preventing infection rather than killing the bacteria may help reduce proliferation of resistant strains of uropathogenic bacteria. It is important to keep confidence of consumers and health care professionals high by providing guidance on selection of efficacious cranberry products. This study provides insights into how the cranberry components used to formulate two popular dietary supplements affect urinary AAA, a potentially important mechanism for preventing UTIs (Ofek et al. 2003; Head 2008).

Results of the fractionation of cranberry fruit and the supplement products demonstrate that the PAC fraction alone exhibited AAA against P-fimbriated *E. coli*, confirming previous results on bioassay-directed fractionation and testing of cranberry

Table 4. Least squares (adjusted) means of the efficacy parameter in healthy participants.

Treatment	Mean	Standard error of mean	95.0% lower conf. limit for mean	95.0% upper conf. limit	DF
WCFD[a]	23.19	2.518	18.23	28.15	259
CJD[b]	94.40	2.518	89.44	99.36	259
Day					
1	50.77	4.314	42.04	59.50	38
2	58.46	4.421	49.51	67.41	38
3	65.38	4.667	55.94	74.83	38
4	61.92	5.299	51.20	72.65	38
5	57.31	5.231	46.72	67.90	38
6	56.15	4.447	47.15	65.16	38
7	61.54	4.499	52.43	70.65	38
Treatment, Day					
WCFD, 1	14.62	6.100	2.27	26.97	38
WCFD, 2	25.38	6.252	12.73	38.04	38
WCFD, 3	31.54	6.601	18.18	44.90	38
WCFD, 4	29.23	7.494	14.06	44.40	38
WCFD, 5	21.54	7.398	6.56	36.52	38
WCFD, 6	16.92	6.289	4.19	29.65	38
WCFD, 7	23.08	6.363	10.20	35.96	38
CJD, 1	86.92	6.100	74.57	99.27	38
CJD, 2	91.54	6.252	78.88	100.00	38
CJD, 3	99.23	6.601	85.87	100.00	38
CJD, 4	94.62	7.494	79.44	100.00	38
CJD, 5	93.08	7.398	78.10	100.00	38
CJD, 6	95.38	6.289	82.65	100.00	38
CJD, 7	100.00	6.363	87.12	100.00	38

[a]AZO Cranberry.
[b]ellura.

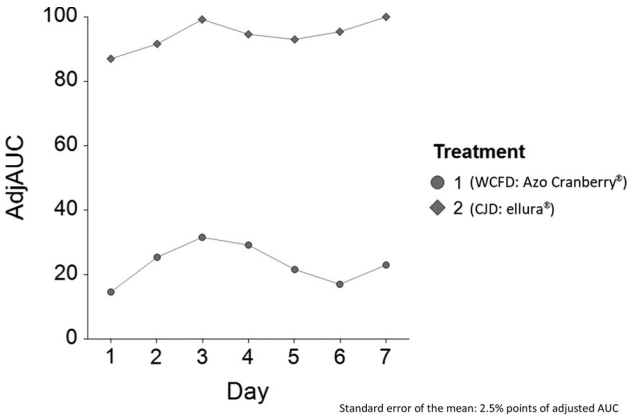


Figure 4. Adjusted AUC over each of seven days for the two cranberry supplement products.

compounds (Howell et al. 1998; Foo et al. 2000a, 2000b; Gupta et al. 2007; Gupta et al. 2012). The high purity of the PAC fraction and high DMAC/A2 levels for soluble PAC content support these findings. The very low residual PAC levels in the flavonol, anthocyanin, acid and sugar fractions did not affect the AAA of these fractions which were all negative. A few previous studies have suggested that other polyphenolic compounds have in vitro AAA (Hotchkiss et al. 2015; Gupta et al. 2016), but fractions containing these compounds may not have been sufficiently purified to remove PACs and/or the compounds may not have been tested at biologically relevant concentrations. The higher

AAA of the CJD PAC fraction versus WCFD may be reflective of differences in PAC size and/or structure and number of A-type linkages (Foo et al. 2000a, 2000b), issues with PAC degradation from harsh processing or storage conditions which can negatively affect bioactivity measurements (Pappas and Schaich 2009), or inaccessibility of the PACs complexed with cellulose fibers and proteins after extraction. The identical AAA of the PAC fractions isolated from CJD and fresh cranberry fruit, suggest that the PACs in CJD were not degraded in processing of the supplement powder.

The differences in composition of the two supplement products were reflected in outcomes of both the in vitro and ex vivo testing. The in vitro AAA results on the whole powders correlate with the soluble PAC levels in each product. Low concentrations of whole CJD powder (0.23 mg/mL) elicited very high AAA, whereas AAA of whole WCFD powder was not detectable at the highest concentration of 60 mg/mL. This is not unexpected since CJD is derived from cranberry juice which only contains soluble PACs. The small percentage (1.9%) of soluble PACs in the Pacran ingredient in WCFD may have been diluted out by the other ingredients in the formulation to the point where no in vitro AAA was able to be detected. The small amount of soluble PAC in WCFD and high levels of insoluble PAC suggests that the product formulation may consist of low concentrations of juice and a higher proportion of whole berry material coming from insoluble pomace, which is typically composed of fruit skins, seeds and stems (Roopchand et al. 2013; Gullickson et al. 2020).

The urinary ex vivo AAA results further support the premise that the soluble PACs are responsible for the activity. The high concentration of soluble PAC per serving in CJD (35.98 mg using DMAC/A2) correlated well with the 94% ex vivo urinary AAA (average of all 24-hr AAA AUCs over all 7 days of supplement administration), while the lower 23% AAA value for WCFD was likely due to the low soluble PAC content per serving (0.94 mg using DMAC/A2). Levels of maximum urinary AAA for each individual product were consistent over the day-to-day period, increasing the reliability of the results obtained over the 1-week intervention period. These positive ex vivo urinary AAA results support previous clinical trials in which cranberry products containing 36 mg of PAC (DMAC/A2) were consumed, resulting in a decrease in UTI recurrence (Uberos et al. 2012; Maki et al. 2016; Occhipinti et al. 2016; Ledda et al. 2017; Thomas et al. 2017).

Results of this study demonstrate that the type of cranberry fruit component used to formulate supplements has a direct impact on in vitro and ex vivo bacterial AAA and could ultimately influence the clinical effectiveness of a product for UTI prevention. A limitation of the current study is that a control group was not included; however, the intention was simply to determine any changes in urinary AAA following intake of supplements containing soluble or insoluble PACs. Previous clinical trials on cranberry supplements that have yielded inconsistent results for recurrent UTI prevention often did not accurately report PAC levels or utilize treatments products with sufficient PAC content (Jepson et al. 2012). The negative trials may have utilized products made from cranberry pomace that contained little or no soluble PACs but did have high levels of insoluble PACs. In our trial, it is improbable that the 19.68 mg/serving of insoluble PACs in WCFD contributed substantively to urinary AAA because previous research has shown that PACs form stable complexes with the cellulose fibers in pomace and do not degrade appreciably during metabolism (Le Bourvellec et al. 2019). However, these

insoluble PAC complexes may influence the gut in other ways by increasing colonic antioxidant activity and reducing intestinal tumorigenesis (Pérez-Jiménez et al. 2013).

Even though more research is needed to determine the relationship between PAC content in cranberry supplements and reductions in UTI in the clinical setting, this study provides useful information on how PAC derived from different parts of the cranberry fruit may influence adhesiveness of urine, an important mechanism of UTI pathogenesis. Understanding that a cranberry juice-based supplement with soluble PAC taken daily provides significantly greater urinary AAA than a pomace-based product containing mainly insoluble PAC could aid consumers in selecting bioactive cranberry supplements for maintenance of urinary tract health.

Declaration of interest

Amy Howell has ownership interest in Complete Phytochemical Solutions, LLC, which performed HPLC and DMAC analyses in this study, and in full disclosure, her affiliation with this company is acknowledged.

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Amy B. Howell is an Associate Research Scientist at the Marucci Center for Blueberry Cranberry Research at Rutgers University in Chatsworth, NJ. Since 1993, she has been engaged in research that targets utilizing cranberry for prevention and management of bacterial diseases, including urinary tract infections (UTIs), stomach ulcers, and periodontal disease. Her primary research focus has been on isolating polyphenolic compounds from cranberry and determining their role in prevention of UTIs and collaborating on ex vivo clinical studies to determine effects of cranberry proanthocyanidins on uropathogenic bacterial adhesion in urine. She studies the pharmacokinetics and bioavailability of the structurally unique cranberry proanthocyanidins in an effort to determine site(s) of action and dose-response. Dr. Howell is involved in method development for powdered cranberry supplements, working closely with regulatory teams from AOAC and USP (US Pharmacopoeia) to determine standard methods for quantification of the bioactive compounds in cranberries.

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