JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibition of Uropathogenic *Escherichia coli* by Cranberry Juice: A New Antiadherence Assay

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A combination of microplate technology and turbidity assessment for testing the adherence of P-fimbriated *Escherichia coli* to human uroepithelial cell line T24, validated with the addition of the known inhibitor 4-*O*-α-D-galactopyranosyl-α-D-galactopyranose (galabiose), resulted in a high-throughput, biologically relevant assessment of cranberry (*Vaccinium macrocarpon*). P-fimbriated ATCC *E. coli* strains 25922, 29194, and 49161 were inhibited by galabiose. ATCC 29194, a representative urine isolate containing the *papGII* allele (Class II fimbrial adhesin) and demonstrating the most significant inhibition in the presence of galabiose, was chosen for further testing. In this assay, a low-polarity fraction of cranberry juice cocktail demonstrated dose-dependent inhibition of *E. coli* adherence. Reported here, for the first time in *V. macrocarpon*, are 1-*O*-methylgalactose, prunin, and phlorizin, identified in an active fraction of cranberry juice concentrate. This in vitro assay will be useful for the standardization of cranberry dietary supplements and is currently being used for bioassay-guided fractionation of cranberry juice concentrate.

KEYWORDS: ***Escherichia coli*; cranberry; *Vaccinium macrocarpon*; uroepithelial; bacterial adhesin; bacterial adhesion; papG protein; P-fimbriae; 1-methoxygalactose; prunin; phloridzin; α-D-galactopyranosyl-(1→4)-α-D-galactopyranose; galabiose; botanical dietary supplement standardization**

INTRODUCTION

Up to a third of all women will experience a urinary tract infection during their lifetime, and the probability increases with age (*1*, *2*). Cranberry juice (*Vaccinium macrocarpon* Ait., Ericaceae), generally in the form of cranberry juice cocktail ("cocktail": a mixture of cranberry juice, water, and sugar), is a popular botanical dietary supplement (*3*, *4*) primarily used for the treatment and prevention of urinary tract infections, with documented human use for at least 55 years (*5*). This use is supported by several clinical trials (*6*−*13*), although some trials, notably for children with neuropathic bladder and catheterization, did not find cranberry to be effective (*14*, *15*). Cranberry juice was originally believed to be active due to its acidifying effect on urine and/or the increased excretion of the cranberry urinary metabolite hippuric acid (*16*, *17*), although later researchers suggested that the bacteriostatic impact from acidification alone could not account for its demonstrated effects (*18*, *19*). Inhibition of adherence of *Escherichia coli* to uroepithelial cells (*20*−*22*), rather than direct bacteriostatic or bactericidal activity, has been proposed as the mechanism of action. Specifically, there is support for inhibition of the papG fimbrial attachment of uropathogenic strains of *E. coli* to human cells (*23*, *24*) by cranberry's A-type proanthocyanidin compounds but not by a B-type dimer or the (−)-epicatechin monomer (*22*, *25*). However, the question has been raised as to whether intact proanthocyanidins can be assimilated in the gut and reach the urinary tract intact (*26*).

A-type proanthocyanidin oligomers contain an extra link (carbon−oxygen) between two of their epicatechin monomer units. The chemically similar B-type proanthocyanidins contain only single, carbon−carbon links between units. Virtually nothing is known about the metabolic route in humans of A-type proanthocyanidins, but evidence currently indicates that B-type proanthocyanidins, especially trimers and larger, are largely degraded in the gut, and/or not assimilated in any quantity, and do not reach the urinary tract intact (*27*−*33*). Whether this information can be applied to A-type proanthocyanidins is unknown. Because the putative active proanthocyanidins are possibly not absorbed and/or may be metabolized before reaching the urinary tract, it is probable that other cranberry compounds or their metabolites are active. An assay using biologically relevant cells and of sufficiently high-throughput

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10.1021/jf052035u CCC: $30.25
Published on Web 10/19/2005

such that it can efficiently guide the fractionation of cranberry is necessary in the search for additional active cranberry compounds. Such an assay can also be used to standardize commercial cranberry products for their antiadherent activity.

Cell-based in vitro assays currently used to assess bacterial attachment to uroepithelial tissue generally use human cells freshly obtained from urine or the human bladder cell line T24 (ATCC HTB-4). Many of these assays determine the inhibition of adherence of organisms such as *E. coli* to these cells by fixing, staining, and counting the attached bacteria individually, using light or scanning electron microscopy, although a few other detection methods are also used (*34*−*37*). Detection of adherence by light microscopy has at least two limitations. First, it is time and labor intensive, thus not practical for bioactivity-guided fractionation. Second, *E. coli* are killed in the process of fixing and staining; thus, this assay cannot distinguish between adhered bacteria that were viable and those that were dead prior to fixing.

Hemagglutination assays are another available method; these test the ability of a bacterial strain to cross-link red blood cells and are used to verify the expression of fimbrial adhesins and to measure inhibition of adherence. Limitations of this method include the fact that while most red blood cells contain the globoside receptor for P-fimbriated *E. coli*, urinary epithelial cells, not red blood cells, are the target of this pathogen in urinary tract infections. In addition, hemagglutination assays are not sufficiently refined to differentiate the three P-fimbrial classes of *E. coli* and are to some degree subjective (*38*). Our experience confirms this latter limitation; we have not found this assay to be sufficiently robust for our purposes.

In consideration of the limitations of the assays described above and to fill an important gap in bioassay-guided fractionation and assessment of commercial cranberry products, the goal of this research was to develop a relatively high-throughput antiadherence assay that uses the biologically relevant human urinary tract T24 cells and papG *E. coli*.

MATERIALS AND METHODS

Bacterial Fimbriae Identification: Polymerase Chain Reaction (PCR) of papGI, II, and III. *PapG* primers were used, and PCR procedures were performed as described by Johnson et al. (*39*) except as follows. Amplification was carried out in a 50 μL reaction mixture containing 10 μL of DNA template, 0.45 μM each primer, and 30 μL of Platinum PCR Supermix (Invitrogen, Carlsbad, CA). The samples of genomic DNA from ATCC *E. coli* strains 10798, 11775, 25922, 29194, 49161, 53498, and 700336 (American Type Culture Collection, Manassas, VA) (common designations K-12, NCTC 9001, DSM 1103, 51-A-266, Abbott 2110, 27, and J96, respectively) were denatured at 95 °C for 7 min. Then, for 10 cycles, DNA was denatured at 94 °C for 1 min, the annealing of primers was at 68 °C for 2 min, and the extension of DNA was at 72 °C for 3 or 5 min. This was followed by 15 cycles of denaturing at 94 °C for 1 min, annealing at 70 °C for 4 min, and extending at 72 °C for 10 min. The samples were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and photographed using a UV illuminator.

Uroepithelial Cells. T24 human uroepithelial cells (ATCC HTB-4) were grown in culture flasks to confluence in Eagle's minimum essential medium (MEM) (Fisher Scientific, Pittsburgh, PA) supplemented with nonessential amino acids (NEAA) (Fisher) and 10% heat inactivated fetal bovine serum (FBS) (Fisher), removed with trypsin/EDTA (1×) (Sigma-Aldrich, St. Louis, MO), and transferred to 96 well microplates (Corning Costar #3997, Fisher), 200 μL per well at approximately 0.5 × 10^6 cells per mL, and again grown to confluence in FBS and NEAA-fortified medium. Immediately preceding the assay, the medium was removed, and wells were carefully washed with 200 μL of saline, fixed with 100% methanol for 2 min, and dried in a laminar flow hood. In our experience, unfixed T24 cells do not adhere to microplates sufficiently for incubations with *E. coli* and repeated washings, necessitating that the cells be fixed prior to assay; no difference was seen in bacterial adherence to remaining T24 cells for a few very carefully conducted experiments comparing fixed with unfixed T24 plates (data not shown).

Bacterial Preparation. Colony forming antigen (CFA) agar was prepared per published protocol (*40*, *41*): 10 g of casamino acids, 1.5 g of yeast extract, 0.05 g of $MgSO_4$, 0.005 g of $MnCl_2$, and 20 g of granulated agar were autoclaved with 1 L of deionized water. *E. coli* strains were grown on blood agar for 24−48 h, subcultured onto CFA agar, and grown at 35 °C for 5−18 h to promote fimbrial expression. Bacteria were transferred to saline, vortexed well, and diluted to between 0.075 and 0.085 AU (1 cm path length), and again diluted 1:10 in saline to an estimated 10^7 colony forming units (CFU) per mL for immediate use.

Galabiose and Globotriose Inhibitors. Galabiose [α-D-galactopyranosyl-(1→4)-α-D-galactopyranose] and globotriose [D-galactopyranosyl-(1→4)-α-D-galactopyranosyl-(1→4)-β-D-glucopyranose], both known to inhibit the adherence of P-fimbriated *E. coli* (*42*, *43*), were purchased from Sigma. Structures were confirmed using NMR techniques (^{1}H, ^{13}C, COSY; 360 MHz Bruker). Ligand was dissolved in saline for a 20 mg/mL (58 mM) stock solution for biological testing.

Vaccinium Fractionation. Two cranberry products were used for this research: cranberry juice cocktail (containing water, cranberry juice, and added sugar) and, in larger quantities for the purpose of fractionation and compound isolation, cranberry juice concentrate ("concentrate"), a product with no added water or sugar. One liter of cocktail, purchased locally, was fractionated over 70 g of Dianon reverse-phase HP-20 resin (Sigma) to remove water and the most polar constituents (e.g., sugars and some nonphenolic organic acids). The retained material was eluted from the column with 100% methanol [high-performance liquid chromatography (HPLC) grade] resulting in a "lower polarity" fraction of fairly polar constituents that was concentrated in flavonoids including anthocyanins, proanthocyanidins, and other compounds including small phenolics. This methanol fraction was dried in vacuo at 37 °C and redissolved in methanol to a stock concentration of 10 mg/mL.

Thirty-two liters of concentrate, obtained from Ocean Spray, Inc., was fractionated as above over 2.2 kg of resin in two lots (12 and 20 L), with 1 L fractions collected throughout a step gradient of 100% deionized water, 20% methanol, 50% methanol, and finally 100% methanol. Samples of all fractions were dissolved in 50−100% methanol for stock solutions of 10 mg/mL for biological testing.

Assay for Galabiose Inhibition. Galabiose samples and saline controls were diluted in a 96 well microplate in NEAA-fortified, citrate-buffered (10 mM, pH 6.0) MEM containing 2.5% methyl-mannose (methyl-α-D-mannopyranoside) (Sigma). The *E. coli*−saline solution described above was added to each well, resulting in a final concentration of approximately 3 × 10^6 CFU/mL bacteria and 1.3−13 mM galabiose in a total volume of 200 μL per well. The *E. coli* sample plate was incubated at room temperature for 30 min. Following incubation, 100 μL per well was transferred to the rinsed, fixed uroepithelial plate, which was then centrifuged at 500*g* for 2 min. The centrifuged plate was incubated in a 5% CO_2 atmosphere at 35 °C for 1 h.

Following the second incubation, medium and bacteria that had not adhered were removed, and wells were gently rinsed with three volumes of 200 μL of saline. Medium (200 μL, RPMI-1640 with L-glutamine) (Fisher) fortified with 5% FBS was added to each well. Care was taken not to create bubbles in all transfers and mixing involving FBS-fortified media; bubbles present interfere with optical readings. An *E. coli* standard curve was produced for one empty row of the plate, starting at an estimated 10^7 CFU/mL medium, with doubling dilutions through successive wells. An additional 100 μL of each concentration of the standard curve was diluted in saline, plated on blood agar, and grown overnight to provide CFU verification of bacterial concentration.

The RPMI filled plate was optically read with a microplate reader for baseline (typically 0.05 AU) and then incubated at 35 °C to a mean optical density of 0.150−0.200 AU (4−6 h of incubation time) and reread. Optical densities (and incubation times) outside of these ranges

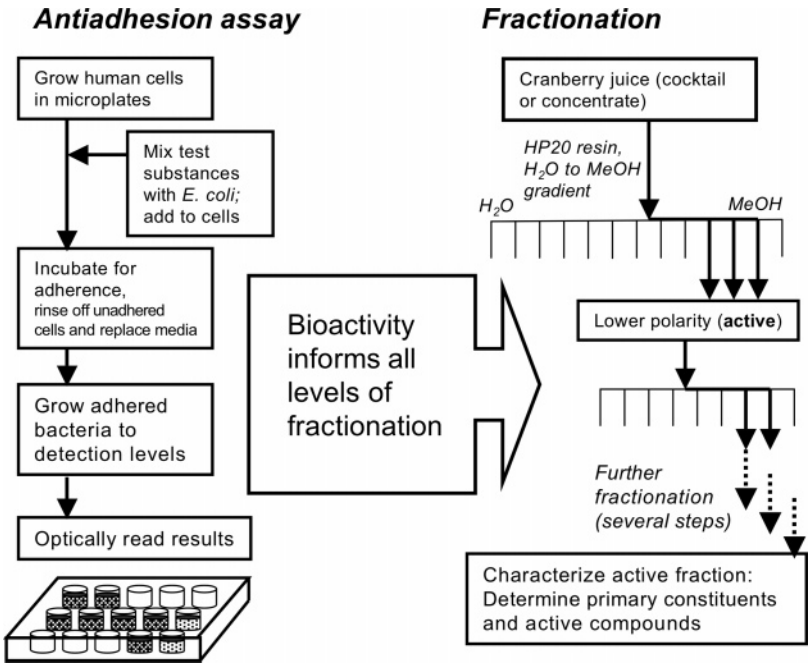

Figure 1. Antiadhesion assay steps and related activity-guided fractionation of cranberry.

do not provide data that is as well-differentiated. Initial concentrations of adherent bacteria were calculated using the standard curve generated; final optical readings, minus baseline, of the standard curve wells were plotted vs initial bacterial concentrations as determined by CFU counts. Inhibition of adherence was determined as the percent difference in the above calculated initial (adherent) bacterial concentrations, comparing test substances with appropriate concentrations of solvent controls (e.g., saline, methanol). Up to three plates were run simultaneously, providing data for 60+ samples and controls, in triplicate, plus a standard curve, in one run. See **Figure 1**.

Galabiose Inhibition in the Presence of FBS. Galabiose was tested, in addition, following the procedure as outlined but with the addition of 10% FBS to the NEAA-fortified, citrate-buffered MEM/methylmannose medium. As above, care was taken with all steps involving FBS-fortified media not to produce bubbles that would interfere with later optical readings.

Inhibition of Adherence by Cranberry Juice Cocktail. The procedure for cocktail was similar to the above methods for galabiose, using the FBS-fortified medium. The lower polarity cocktail fraction from the first-level HP20 separation, described above, was dissolved in methanol to 10 mg/mL and diluted with medium and *E. coli* as above to a final concentration of 50−400 μg/mL. Control wells contained medium, *E. coli*, and, importantly, an equivalent volume of methanol. (Solvents, whether methanol, ethanol, or DMSO, are generally bactericidal to some degree, so precise solvent controls are critical; we have not found DMSO to be a suitable solvent for the lower polarity cranberry fractions.) Cranberry concentrate and fractions thereof were also tested using this method.

Parallel Antibacterial Check. To determine whether test substances had antibacterial (bacteriostatic or bactericidal) activity, rather than antiadhesive activity that left bacteria viable, a parallel plate was incubated containing 10 μL of the above sample medium−*E. coli* solution (prior to addition to the uroepithelial plate), carefully mixed with 190 μL of FBS-fortified RPMI per well. The plate was incubated following the same protocol as above (35 °C for 4−6 h), and optical readings were recorded. Additionally, minimum inhibitory concentrations (MICs) were determined for the known cranberry constituent benzoic acid (Sigma) by traditional methods: medium (MEM) or urine was titrated to pH 5.0, 6.0, and 7.0, and 100 μL was added to wells of a microplate. Benzoic acid was dissolved in medium or urine and titrated to matching pH. A 100 μL volume was then added to initial wells, with serial dilutions for final concentrations of 256, 128, 65, and 0 μg/mL. A 100 μL volume of *E. coli*, at 10^3 CFU/mL in media or urine with equivalent pH, was added to each well, and plates were incubated at 35 °C and examined for evidence of growth after 20−24 h.

HPLC Detection of Benzoic Acid. The concentrate was separated on a 200 mm × 20 mm 5μ ODS-4 HE GROM-SIL 120 preparative column (Watrex, San Francisco) with a mobile phase gradient of methanol:0.5% TFA in H_2O (10:90, v/v), changing to 90:10 over 30 min, and held at 90:10 for 20 min, with PDA detection at 200−400 nm.

NMR Confirmation of Known Ligands. Structures of the known ligands galabiose and globotriose were confirmed using NMR techniques (^{1}H, ^{13}C, COSY; 360 MHz Bruker).

Safety. *E. coli* strains, transformed T24 uroepithelial cells, and human urine are biological hazards and were handled and disposed of using approved protocols. Urine was obtained from volunteers in accordance with an IRB-approved protocol. Chemical reagents were likewise handled according to standard laboratory safety protocols.

Statistical Analysis. Calculations were accomplished with Microsoft Excel 2004 for Mac. Standard curves were generated using *n*th-order polynomial fit with $r^2 > 0.97$. *P* was calculated using a one-tailed *t*-test for heteroscedastic data and was considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Identification of *E. coli* Fimbrial Class. The PCR of the *E. coli* strains demonstrated that strain 700336 (isolated from a human patient with pyelonephritis) possessed the Class I allele of *papG*, as well as the Class III allele, in agreement with published data; Class I is rarely seen in clinical isolates (*44*). Other strains with the Class III allele were 49161 and 53498 (both isolated from urine). Strains 25922 ("clinical isolate") and 29194 (urine) had the Class II allele. PapG alleles were not detected for ATCC strains 10798 (human feces, diphtheria convalescent) or 11775 (urine). Other fimbriae typically present on *E. coli* include type 1; we did not examine strains for the allele responsible for this fimbrial class but inhibited any type 1 activity with 2.5% methyl mannose (*45*).

Identification of Known Ligands. NMR verification was considered necessary because commercially available natural products are occasionally of very low purity (*46*); additionally, the stereochemistry of these ligands is essential for binding. Galabiose was determined to consist of 4-*O*-α-D-galactopyranosyl-α-D-galactopyranose and 4-*O*-β-D-galactopyranosyl-α-D-

Table 1. Galabiose Inhibition of Adherence of Seven *E. coli* Strains to Uroepithelial T24 Human Bladder Cell Line in FBS-Free Medium[a]

	E. coli strains: fimbrial class and adherence inhibition				
ATCC strain	isolated/source	P fimbrial class	relative binding avidity	inhibition, 3.9 mM galabiose (%)	*P*
10798 (K-12)	feces from diphtheria convalescent	ND	very high	1	NS
11775	urine	ND	moderate	58	0.021
25922	clinical isolate	II	moderate	25	0.039
29194	urine	II	high	44	0.001
49161	urine, North Carolina	III	low	42	0.009
53498	human urinary tract infection	III	moderate	7	0.061 (NS)
700336 (J96)	human/ pyelonephritis	I, III	moderate	7	NS

[a] NS, not significant; ND, *papG* alleles not detected.

galactopyranose in a ratio of 64:100, respectively, calculated on integration of the 5′-proton signals at 4.02 to 4.35 ppm. A ratio of α- and β-forms is expected for anomeric sugars when in aqueous solution. Globotriose was confirmed by NMR to be a mixture of the α- and β-anomers, also as expected.

Inhibition of *E. coli* Strains by Known Ligands. An *E. coli* strain suitable for activity-guided fractionation of cranberry concentrate and for testing of cranberry products was chosen based on its adherence and inhibition in the presence of the known inhibitor galabiose. Both of the Class II and one of the Class III P-fimbriated strains tested, ATCC 25922, 29194, and 49161, were inhibited from adhering to T24 human uroepithelial cells by galabiose at 3.9 mM (25, 44, and 42%, respectively) (**Table 1**). Another strain, ATCC 53498 (Class III), only trended toward being weakly inhibited, and inhibition was not seen for ATCC 700336 (Classes I and III). These two latter strains may have other adhesins that are not inhibited by methyl-mannose or galabiose, or they may require the higher inhibitory activity of globotriose. Non-P-fimbriated strain ATCC 10798, a fecal isolate that bound avidly, was not affected by the presence of galabiose. Interestingly, ATCC 11775, a strain for which we did not detect P-fimbrial alleles, was inhibited. While inhibition of this strain is not supportive of the proposed mechanism for this assay, it is possible that there are undetected fimbriae that are nonetheless inhibited by galabiose or that other factors were involved; further testing of this strain is warranted. Relative binding avidity was designated as follows: low adherence, less than 2×10^6 CFU/mL; moderate adherence, 3×10^6 to 6×10^6 CFU/mL; high adherence, 7×10^6 to 15×10^6 CFU/mL; very high/avid binding, greater than 20×10^6 CFU/mL. Concentrations of bacteria are based on 200 μL wells, with bacterial adherent to confluent uroepithial cells on the bottom surface. We chose ATCC strain 29194, the strain with the highest adherent activity that was also significantly inhibited by galabiose, as the most suitable strain for further work with cranberry products.

Addition of FBS to the incubating medium reduced the sensitivity of this assay substantially, but inhibition was still seen for high concentrations of galabiose (**Figure 2**): 6.5 mM provided 17% inhibition and 13 mM [10 times the published IC_{50} for this compound under different conditions (*42*)] produced a 26% inhibition ($p = 0.033$ and 0.006, respectively; $n = 3$).

The known ligand globotriose also inhibited adherence of ATCC strain 29194 in FBS-free medium, at concentrations less

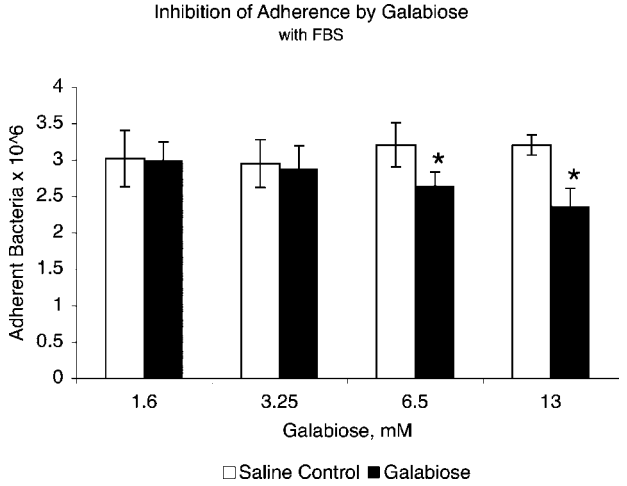

Figure 2. Inhibition of adherence by galabiose in FBS-fortified medium; $*p < 0.05$.

than one-sixth that of galabiose: 2.3 mM galabiose and 0.35 mM globotriose both provided 77% inhibition relative to saline control ($n = 3$, $p = 0.022$). There were slight differences in methods used as compared to those stated above (there was no citrate buffer, and T24 plates were incubated with MEM after fixing and before sample and *E. coli* addition). Because different parameters were used, precise comparison with other galabiose runs is not possible. Nonetheless, it is clear that globotriose is a substantially superior, if expensive, inhibitor. These data are consistent with data published by others (*42*, *43*). Because of the prohibitive cost of globotriose, as well as sporadic availability, all subsequent experiments were conducted with galabiose. Some larger oligomers in this class produce even higher inhibition of P-fimbrial adherence (*47*, *48*), but these are not available commercially and were not tested.

One experiment was run in larger, six well microplates to accommodate a light microscope lense, with corresponding increases in all cell and reagent volumes (total 2.75 mL per well). The wells were fixed and stained following the final rinse and examined by light microscopy for total number of adhered bacteria on a mean of at least 200 uroepithelial cells. Galaboise (3.9 mM) inhibited adherence by 92% ($p < 0.001$) as compared to saline control, providing direct visual support for our method. No further runs were conducted using this labor intensive method. This experiment was conducted without fixing the T24 cells (direct examination of adherent *E. coli* per uroepithelial cell eliminates this need) and using 100 mM citrate buffer at pH 5.0. Therefore, as with the globotriose data, exact comparisons of percent inhibition with other data reported here are not appropriate. We have found that several parameters can be varied in our method still providing effective results; however, the percent inhibition will vary. While we have not done an exhaustive study of all variations, the parameters reported in the Materials and Methods (pH, citrate buffer concentration, concentration of *E. coli*, etc.) have provided the best results for testing of both cranberry fractions and known globoside ligands.

Inhibition by Cranberry Juice Cocktail. We have found that the testing of more complex substances (e.g., fractions of concentrate and cocktail) requires the addition of FBS to the medium. Addition of FBS may be necessary for several reasons, and we suggest two. First, FBS will provide some buffering effect, and we have seen that pH shift (a color shift of the MEM pH indicator) is less erratic when in the presence of FBS. While pH is probably an important, although not essential, factor in the activity of cranberry products against urinary tract infections,

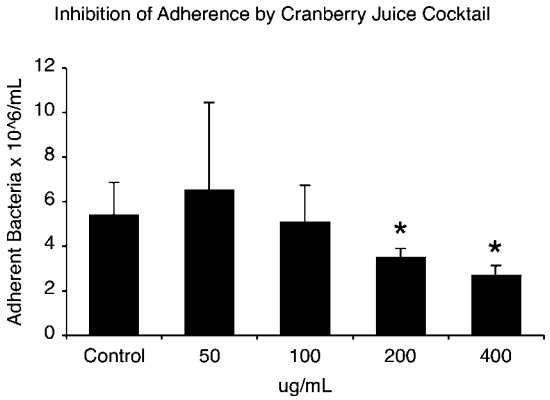

Figure 3. Inhibition of *E. coli* 29194 adherence by a lower polarity fraction of cranberry juice cocktail; *$p < 0.05$ vs control.

stabilization of pH by FBS removes this complicating variable in the current assay for adherence. Second, and possibly more important, FBS provides sufficient nutrients that substantially increase the growth rate of *E. coli* over its rate in unfortified medium (data not shown). Without FBS, complex mixtures such as cranberry juice likely provide substantial nutrients to minimal media, resulting in a higher rate of *E. coli* growth in cranberry wells as compared to controls and leading to the mistaken impression that cranberry is increasing adherence. The addition of sufficient FBS ensures that bacteria are growing at or near their maximum growth rate, in both verum and control wells, throughout the assay. Additions of complex mixtures (e.g., cranberry) do not increase nutrient levels or growth rate significantly if FBS is present; thus, a major confounding factor is eliminated. However, as seen with galabiose, the addition of FBS also lowers sensitivity, requiring a substantial increase in ligand concentration to see an equivalent inhibition. This may be due to the large concentration of added soluble protein, interfering to some extent with ligand−receptor interactions.

We have therefore tested complex mixtures (cocktail, concentrate, and fractions thereof) at higher than typical concentrations in the presence of FBS and have found in particular that lower polarity fractions of cocktail consistently demonstrate inhibition in our assay (**Figure 3**). Fifty percent inhibition was seen at 400 μg/mL. It is hypothesized that inhibition with individual, nonnutritive compounds from this mixture will test positive at significantly lower doses in unfortified medium, as was seen for galabiose.

The concentrate produced similar results; to date, we have identified three compounds from an active fraction (**Figure 4**) previously unknown to cranberry: Five milligrams of 1-*O*-methylgalactose (**1**) was obtained, corresponding to 4.9 ppm in cranberry juice concentrate (w/w, including water weight of concentrated juice). Data for (**1**), 1-*O*-methylgalactose: λ_{max} (ϵ) (MeOH): 207 nm (1301). IR (dry film): ν_{max} 3370, 1076 cm^{-1}. ^{1}H NMR (CD_3OD, 360 MHz): δ 4.13 ppm (1H, d, J = 7.4 Hz, H-1), 3.83 (1H, dd, J = 1.0, 2.9 Hz, H-4), 3.77 (1H, dd, J = 6.6, 11.4 Hz, H-6a), 3.72 (1H, dd, J = 5.4, 11.4 Hz, H-6b), 3.53 (3H, s, OMe), 3.51 (1H, dd, J = 1.0, 9.7 Hz, H-3), 3.50 (1H, dd, J = 7.4, 9.7 Hz, H-2), 3.48 (1H, m, H-5). ^{13}C NMR (CD_3OD, 360 MHz): δ 105.8 ppm (C-1), 72.3 (C-2), 76.5 (C-3), 70.1 (C-4), 74.8 (C-5), 62.3 (C-6), 57.1 (OMe). NMR data are consistent with those published by others (*49*−*54*). A 5.2 mg amount of prunin (**2**) was obtained, corresponding to 1.6 ppm in cranberry juice concentrate (w/w). Data for prunin (**2**): λ_{max} (ϵ) (MeOH): 215 (7731), 275 nm (2069). IR (dry film): ν_{max} 3365, 1684, 1646, 1200, 1064 cm^{-1}. ^{1}H NMR (CD_3OD, 360 MHz): δ 7.34 ppm (2H, d, J = 8.8 Hz, H-2′, H-6′), 6.83 (2H, d, J = 8.8 Hz, H3′, H5′), 6.22 (1H, d, J = 2.2 Hz, H-8), 6.19 (1H, d, J = 2.2 Hz, H-6), 5.39 (1H, dd, J = 3.0, 13.0 Hz, H-2), 4.98 (1H, d, J = 7.3 Hz, H-1″), 3.88 (1H, dd, J = 2.2, 12.1 Hz, H-6″a), 3.68 (1H, dd, J = 5.3, 12.1 Hz, H-6″b), 3.46 (1H, overlapped, H-3″), 3.46 (1H, overlapped, H-5″), 3.45 (1H, overlapped, H-2″), 3.42 (1H, overlapped, H-4″), 3.18 (1H, dd, J = 13.0, 17.2 Hz, H-3a), 2.75 (1H, dd, J = 3.0, 17.2 Hz, H3b). ^{13}C NMR (CD_3OD, 360 MHz): δ 80.8 ppm (C-2), 44.3 (C-3), 198.7 (C-4), 164.7 (C-5), 98.1 (C-6), 167.1 (C-7), 97.0 (C-8), 165.1 (C-9), 105.0 (C-10), 131.0 (C-1′), 129.2 (C-2′), 116.4 (C-3′), 159.2 (C-4′), 116.4 (C-5′), 129.2 (C-6′), 101.3 (C-1″), 74.8 (C-2″), 78.4 (C-3″), 71.1 (C-4″), 77.9 (C-5″), 62.4 (C-6″). NMR data are consistent with those published by others (*55*−*58*). Data for phloridzin (**3**) (3.9 mg obtained, equivalent to 1.2 ppm w/w). λ_{max} (ϵ) (MeOH): 215 (4287), 277 nm (1284). IR (dry film): ν_{max} 3366, 1684, 1628, 1204 cm^{-1}. ^{1}H NMR (CD_3OD, 360 MHz): δ 7.07 ppm (2H, ddd, J = 2.2, 3.0, 8.3 Hz, H-2, H-6), 6.69 (2H, ddd, J = 2.2, 3.0, 8.3 Hz, H-3, H-5), 6.19 (1H, d, J = 2.1 Hz, H-3′), 5.95 (1H, d, J = 2.1 Hz, H-5′), 5.04 (1H, d, J = 7.2 Hz, H-1″), 3.91 (1H, dd, J = 2.0, 12.0 Hz, H-6″a), 3.72 (1H, dd, J = 5.3, 12.0 Hz, H-6″b), 3.46∼3.47 (3H, overlapped, H-2″,3″,5″), 3.44 (2H, t, J = 7.5 Hz, 2H-α), 3.42 (1H, m, H-4″), 2.88 (2H, t, J = 7.5 Hz, 2H-β). ^{13}C NMR: δ 134.0 ppm (C-1), 130.5 (C-2, C-6), 116.5 (C-3, C-5), 156.5 (C-4), 206.6 (C═O), 47.1 (C-α), 30.9 (C-β), 106.9 (C-1′), 162.4 (C-2′), 95.5 (C-3′), 166.0 (C-4′), 98.4 (C-5′), 167.7 (C-6′), 102.2 (C-1″), 74.8 (C-2″), 78.6 (C-3″), 71.2 (C-4″), 78.5 (C-5″), 62.5 (C-6″). NMR data are consistent with those published by others (*59*, *60*).

These three constituents were found in an active subfraction of the low-polarity concentrate fraction. 1-*O*-Methylgalactose (**1**) was obtained in sufficient quantities for testing in the described assay but did not demonstrate antiadherent or antibacterial activity. Nonetheless, the presence of one *O*-methyl sugar in the active fraction suggests that others may be present. In view of the fact that 1-*O*-methylgalabiose has nearly 10 times the inhibitory effect of galabiose (*42*), these sugars are good targets for activity.

Parallel kill-check plates run for all galabiose testing did not detect any bactericidal or bacteriostatic activity. We have seen cocktail fractions produce minimal antibacterial activity but at a rate lower than the antiadhesion activity described above (data not shown). Benzoic acid, a small cranberry phenolic retained in our first-level fractionation and a known antibacterial, is present in samples of concentrate at approximately 0.3% dry weight (final extracted weight to estimated dry mass of initial volume of concentrate) and may account for the antibacterial activity seen. The identity of benzoic acid isolated from the low-polarity fraction of cranberry concentrate was confirmed with ^{1}H NMR (data not shown). Benzoic acid was also easily

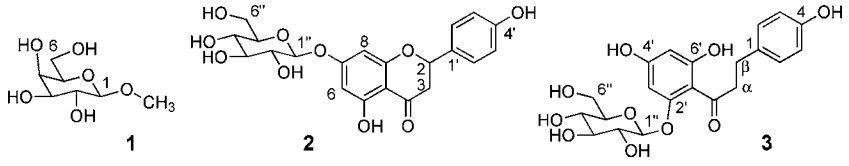

Figure 4. 1-*O*-Methylgalactose (**1**), prunin (**2**), and phloridzin (**3**).

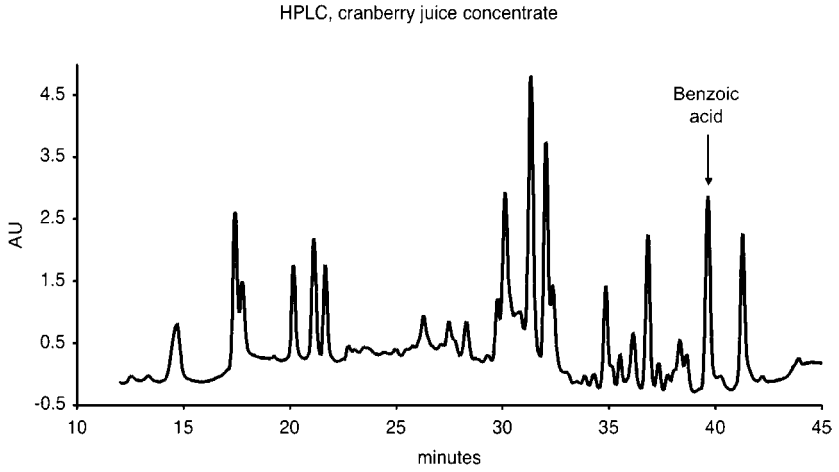

Figure 5. HPLC chromatogram of cranberry juice concentrate.

detectible in whole concentrate by HPLC without prior purification or fractionation (**Figure 5**). Because this antibacterial constituent is generally present in considerable amounts in cranberry products, it is important to verify whether any cranberry activity seen in an assay is inhibition of adherence or more generally the killing of bacteria. Several antiadherence studies done to date (*18*−*20*, *61*−*63*) do not clearly state whether they have distinguished between these two activities.

Because benzoic acid is present in the fractions of cocktail and concentrate that have tested positive in our assay, some of the "antiadherent" activity that we have seen could be due, instead, to its bacteriostatic or bactericidal activity. In separate MIC tests, *E. coli* growth in benzoic acid-free urine was not inhibited at pH greater than 4.5 but was limited by the addition of benzoic acid at higher pH levels: MICs at pH 5.0 and 6.0 were below 64 μg/mL. At pH 7.0, inhibition was seen only at 256 μg/mL benzoic acid. Growth in MEM gave similar results. To assess the activity of our low-polarity concentrate fraction without the complication of antibacterial action from benzoic acid, we used HSCCC methods (currently being refined) to remove this compound without substantially changing the remaining metabolome. Inhibition, but no bactericidal/bacteriostatic activity, was seen in the resulting benzoic acid-free fraction, thus confirming the presence of antiadherent compounds. The compounds 1-*O*-methylgalactose, prunin, and phloridzin were found in this active fraction. Although not active in isolation, these three compounds help to characterize this active fraction; they may have no activity whatsoever or may be involved in synergistic combination with other compounds present.

It should be noted that the purified active fraction is still very complex. On the basis of HPLC analyses of the active fraction and the above three compounds, we conclude that there are at least 245 additional constituents at the level of 20 ppm or below. This is not unexpected. Botanicals and fractions thereof are highly complex, consisting of hundreds if not thousands of compounds, and a search for a single active constituent is often not practical nor, in the case of synergism, even possible. Standardization based on the characterization of active fractions with a few compounds, even if these are inactive in isolation, is the best that current technology affords for the majority of botanical dietary supplements, including cranberry.

We recognize some limitations to this assay and note that the following studies would strengthen the argument for its validity: (i) Additional work using P-fimbrial Classes I, II, and III recombinant strains of *E. coli*, plus their hosts and wild strains, and further assessment of anomalous ATCC strain 11775, would help to confirm that this assay is measuring inhibition of P-fimbrial attachment specifically; (ii) Western blotting or other methods could be used for direct examination of P-fimbrial expression after *E. coli* growth on CFA agar, supporting our PCR assessment of genotype; (iii) a comparison of the degree to which a series of *E. coli* strains are inhibited by galabiose, with the same strains' inhibition by cranberry fractions would, if a correlation is seen, further support claims that cranberry works via P-fimbrial inhibition; and (iv) The complicating effect of FBS could be further illuminated in experiments holding all other parameters constant.

Additionally, further studies using the more expensive trisaccharide globotriose, which has a higher activity and may be more important in the case of Class III P-fimbriae (*64*), could reveal activity for Class III ATCC strains 53498 and 700336. Other types of adherence could potentially be assessed using the methods presented here: for example, by holding galabiose (or globotriose) concentrations constant and varying methylmannose, it should be possible to measure type 1 fimbrial adhesion.

In conclusion, this assay is designed as high-throughput in vitro approximation of human urinary tract infection, using both uropathogenic (P-fimbriated) *E. coli* strains and the T24 human bladder cell line, and adapted to microplate technology with relatively fast optical detection. This method will accommodate the testing of multiple commercial products at varying concentrations and thus can be utilized for standardization as well as fractionation purposes. The active cranberry compounds found to date by others, A type proanthocyanidins, are possibly not excreted intact in the urine; therefore, testing of other cranberry compounds and their urinary metabolites will be beneficial and can be accomplished with the method described here. This method has been validated with known P-fimbrial ligands, demonstrated dose-dependent inhibition of adhesion with cranberry juice cocktail, and demonstrated activity for cranberry before and after removal of the antibacterial compound benzoic acid. Activity-guided fractionation using these methods has led to the discovery of 1-*O*-methylgalactose, prunin, and phloridzin, previously not known to occur in cranberry.

ABBREVIATIONS USED

CFU, colony forming units; FBS, fetal bovine serum; galabiose, α-D-galactopyranosyl-(1→4)-α-D-galactopyranose; globotriose, D-galactopyranosyl-(1→4)-α-D-galactopyranosyl-(1→4)-β-D-glucopyranose; MEM, Eagle's minimal essential

medium; MIC, minimal inhibitory concentration; NEAA, nonessential amino acids; RPMI, RPMI-1640 medium.

ACKNOWLEDGMENT

We thank Ocean Spray Cranberries, Inc. for the cranberry juice concentrate used in this study and for an unrestricted gift.

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Received for review August 18, 2005. Accepted August 23, 2005. Research was generously supported by Grant P50AT00155 and an NRSA Fellowship for A.T. (F3AT00623) from the National Center for Complementary and Alternative Medicine (NCCAM), the Office of Dietary Supplements (ODS), the Office for Research on Women's Health (ORWH), and the National Institute of General Medical Sciences (NIGMS). The contents are solely the responsibility of the authors and do not necessarily represent official views of NCCAM, ODS, ORWH, or NIGMS.

JF052035U