

Antimicrobial Activity of *Vaccinium macrocarpon* (Cranberry) Produced Proanthocyanidin (PAC) on the Growth and Adhesion Properties of *Staphylococcus aureus*

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Abstract

Cranberries have long been used by lay people to relieve the symptoms of urinary tract infections. Recent research has determined that the component of cranberry called proanthocyanidin (PAC) is the primary mechanism for inhibiting P-fimbriated *E.coli* adhesion to uroepithelial cells *in vitro*. A series of experiments were performed to determine the effects of PAC on growth and adhesion of uropathogenic *E. coli* and *Staphylococcus aureus* to urinary catheter material. The results showed that PAC-inhibited binding of Gram positive *S. aureus* to collagen coated surfaces and significantly decreased the growth of these bacteria. P-fimbriated *E.coli* did not bind well to the biomaterial and their growth was unaffected by the cranberry extract with the exception of some loss in viability at 1000 µg/mL after 5 to 18 hours of exposure. This is the first report of the potential for cranberries to interfere with the adhesion and growth of *S. aureus*, a multi-drug resistant organisms responsible for morbidity and mortality especially in hospitalized patients.

Key words: P-fimbriated *Escherichia coli*, *Staphylococcus aureus*, bacterial adhesion, cranberry, proanthocyanidin, urinary tract infection

INTRODUCTION

Cranberries, in juice or solid form, have long been used as a natural food remedy for people suffering from urinary tract infections (UTI). Early studies suggested that the beneficial effects of cranberries were due to their ability to acidify the urine, and that hippuric acid was the likely anti-microbial factor (1). However, more recent studies suggest that it is the 'condensed tannins' of cranberries that are active agents responsible for its anti-microbial effects, and that mechanistically these compounds exert their beneficial effects by inhibiting the binding of uropathogens to host cells within the urinary tract (2).

Escherichia coli are the major cause of uncomplicated UTI and while they possess a number of virulence factors, it is generally believed that two types of fimbriae are the primary mediators of the initial stages of the disease process. Type 1 fimbriae adhere to mannose moieties on the vaginal, urethral and bladder surfaces and appear to be important in cases where the pathogens invade the cells (3,4), while type P fimbriae attach to glycolipid receptors (5). P-fimbriated *E. coli* are the major

cause of pyelonephritis and as urinary catheters and stents can enhance ascension of associated bacteria to the kidney, the ability of compounds to inhibit their binding is important in these patient populations.

Cranberries contain two or three compounds which could have anti-infective properties. Fructose can inhibit the adhesion of type 1 fimbriated *E. coli* to uroepithelial cells (6). Of more importance are the condensed tannin proanthocyanidins (PAC) which competitively inhibit the attachment of type P-fimbriated *E. coli* (7,8). Of these condensed tannins, three A-type PAC trimers from cranberries have been shown to be critical to blocking pathogenic *E. coli* adhesion to bladder cells (8). However, little is known about the physio-chemical properties of complex flavonoid PAC (Fig. 1) or the other tannins found in various cereals and legume seeds.

Two studies have shown the potential for cranberry juice consumption by spinal cord injury patients to reduce the extent of Gram negative (e.g. *Escherichia coli*) and Gram positive (e.g. *Staphylococcus aureus*) bacterial biofilms in the bladder (9,10). The effect of cranberries on Gram positive bacteria has not been previously documented or studied and therefore the goals of this study

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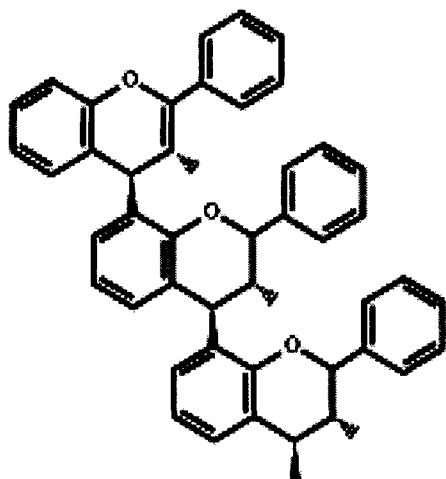


Fig. 1. Structure of proanthocyanidin.

were to assess whether PAC could inhibit the growth and adhesion of *S. aureus* to biomaterial surfaces.

MATERIALS AND METHODS

Bacterial culture conditions

P-fimbriated *E. coli* HU357 was initially streaked on a CFA (colonization factor antigen) agar plate and stored at 4°C in order to maintain the P-fimbriated phenotype of the bacteria. *S. aureus* was streaked on BHI (brain heart infusion) agar plates and stored at 4°C. These plates single colonies were used to inoculate small (3 mL) cultures of bacteria using either CFA (*E. coli*) or BHI (*S. aureus*) broth.

Proanthocyanidins

PAC crystals were obtained from Dr. Amy Howell at the Philip E. Marucci Center for Blueberry and Cranberry Research of Rutgers University (New Jersey, USA). The PAC was reconstituted in distilled water at a concentration of 50 mg/mL and stored in small aliquots (0.5 mL) at -20°C.

Adhesion assay

Twelve well plates containing circular 18 mm glass microscope coverslips were precoated with 0.25 mg/mL of collagen type I for one hour at room temperature, to better simulate an *in vivo* environment. Glass coverslips were then washed 2x with a phosphate buffered saline (PBS) solution. Bacteria that were exposed to varying concentrations of PAC (1 hr, 37°C, in liquid culture) were then added to each well (10^6 /well) and incubated for 1 hr at 37°C to facilitate binding to the collagen coated surfaces. PAC concentrations of 1000 µg/mL, 50 µg/mL and 25 µg/mL were used because these concentrations can be safely ingested and are below biocidal levels. The media was then removed from each well and the coverslips

gently washed 2x with PBS. Adherent bacteria were then fluorescently stained using a BacLight™ staining kit according to the manufacturer's instructions (Molecular Probes, USA). Coverslips were removed and mounted onto glass slides using DAKO aqueous mounting media (DAKO Inc. CA, USA). Images were taken using a 60x Apo Nikon inverted fluorescent microscope. Digital fluorescent images were acquired on a Nikon Eclipse TE200 inverted microscope (Nikon Pan Apo 60x) using a liquid cooled CH350 CCD camera (Photometrics Ltd., Tucson AZ). Images were only manipulated to enhance the contrast and brightness.

Growth assay

PBS containing either *E. coli* (10^6 /mL) or *S. aureus* (10^6 /mL) were supplemented with either 0.5% or 5% of either CFA (*E. coli*) or BHI (*S. aureus*). These defined culture conditions were chosen based on their growth promoting (5%) and short-term survival (viable but 0% growth) properties. In this way we could differentiate between any potential bactericidal or bacteriostatic effects of PAC. Preliminary experiments showed that PBS supplemented with 0.5% media produced zero-growth, while PBS supplemented with 5% media promoted bacterial growth. Using these defined conditions 20 mL cultures of *E. coli* and *S. aureus* were supplemented with 1000 µg/mL PAC. At specified time points 1 mL aliquots were collected and used to measure both bacterial concentration (spectrometrically at OD 600) and viability (colony forming units: CFU) by plating on either LB (for *E. coli*) or BHI (for *S. aureus*) agar plates and incubating overnight at 37°C. Experiments were carried out in duplicate and values presented at mean +/- standard deviation (SD).

RESULTS

Inhibition of adhesion and growth

Glass coverslips inoculated with PAC and treated *S. aureus* showed noticeably less bacterial adhesion than the PBS control (Fig. 2) ($p < 0.01$). Analysis of 10 fields of view per sample indicated four fold less adhesion when treated with 1000 µg/mL PAC concentrations and two to three fold less adhesion after use of lower concentrations of PAC. Adhesion of *E. coli* was extremely poor and no differences were observable between PAC treated samples and controls.

There was significant reduction in growth of *S. aureus* in the presence of 1000 µg/mL PAC (Fig. 3). This was not due to pH, which remained neutral throughout all assays. Optical density data do not measure bacterial viability per se, but CFU counts confirmed the reduction in staphylococcal growth (Table 1).

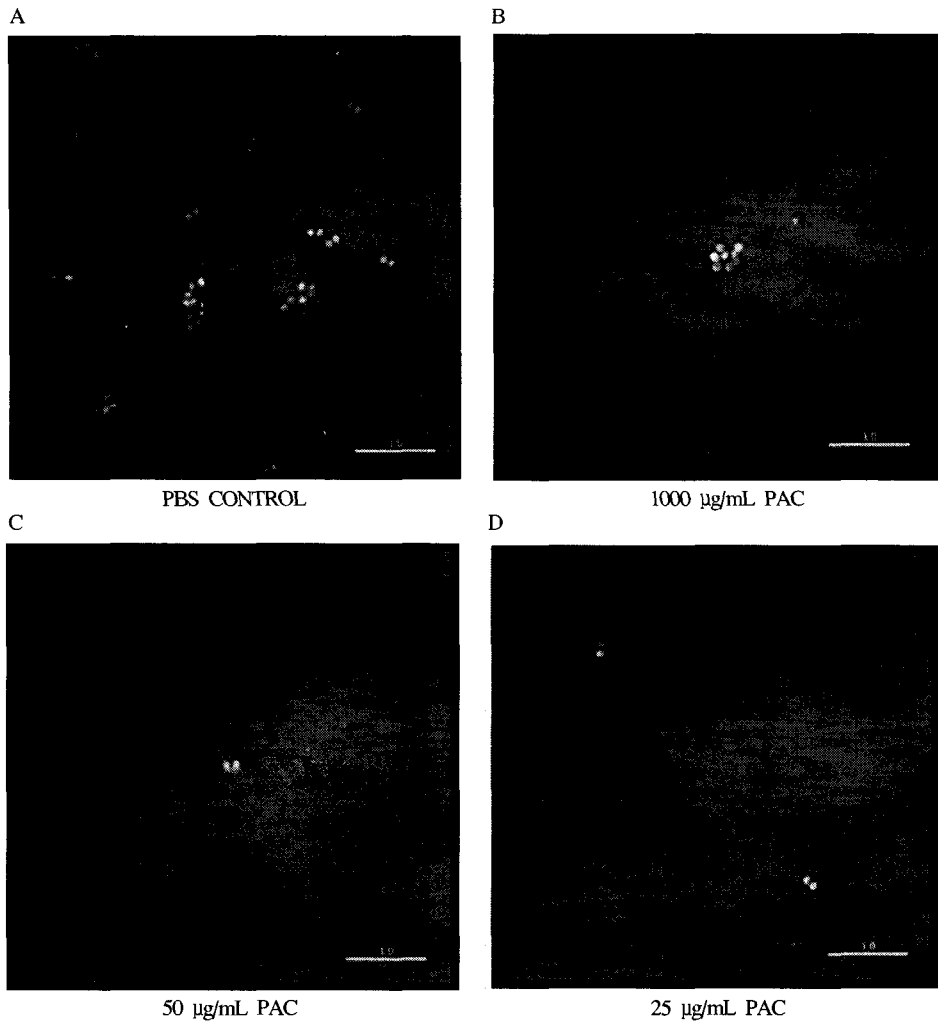


Fig. 2. Effects of PAC on *S. aureus* adhesion. Fewer adherent bacteria (arrows) were noted on surfaces treated with proanthocyanadins, especially at the higher dose, although no linear concentration correlation could be determined by this observational technique.

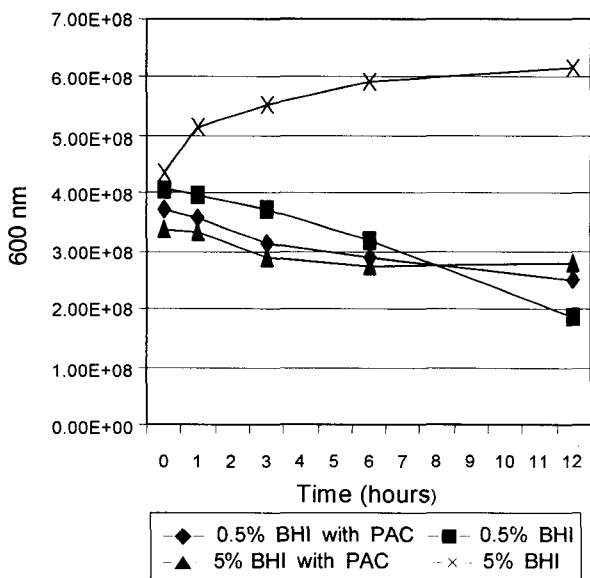


Fig. 3. *S. aureus* growth with 1 mg/mL PAC in 0.5% and 5% BHI media.

Table 1. Comparisons between *S. aureus* grown in a concentrations of 1000 µg/mL PAC and standard bacteria growth in PBS

	0 h	1 h	3 h	6 h	12 h
0.5% BHI	2000	834	487	292	167
0.5% BHI with PAC	0.5	0	0	0	0
5% BHI	117	630	433	893	330
5% BHI with PAC	2	0	0	0	0

S. aureus was grown in 0.5% CFA media (which does not support their growth) and 5% CFA media (which does allow some growth and therefore acts as a test of inhibition of active metabolism). The findings show significant killing of the bacteria by the PAC.

The combined data show that the inhibitory activity appears to occur while the organisms are in planktonic form, otherwise adhesion would not have been affected and dead bacteria would have been found on the surfaces. This could imply that an adhesive component of the staphylococci was modified in the presence of PAC,

thereby reducing adhesion to the surface. The inhibitory effect occurs within the first hour or exposure of the bacteria to the PAC, suggesting it does not require a growth cycle to take effect, unlike for example penicillins.

P-fimbriated *E. coli* grown in proanthocyanidin extract showed no ability to grow in PAC under the 0.5% media conditions, and no significant loss of viability from controls when incubated in 5% CFA, except with 1000 $\mu\text{g}/\text{mL}$ PAC when there was a 66% drop in viable cell count between 5 and 18 hours incubation (Fig. 4).

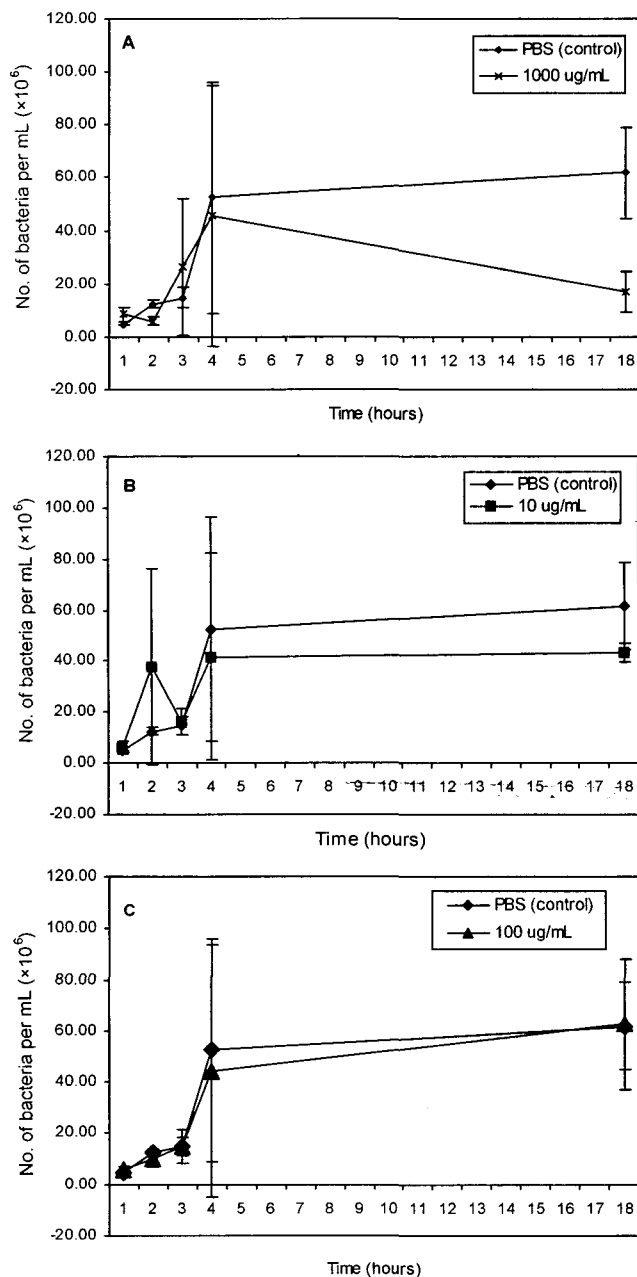


Fig. 4. Growth counts of *E. coli* in the presence of 1000 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$ proanthocyanidin compared to addition of phosphate buffered saline (PBS). The *E. coli* growth is only reduced at 18 hours in 1000 $\mu\text{g}/\text{mL}$.

DISCUSSION

There is evidence that cranberry juice may decrease the number of symptomatic UTIs over a 12 month period in women due to interference with adhesion of *E. coli* to cells (11). The present findings are the first to show that proanthocyanadins are able to affect the growth of uropathogens and decrease the adhesion of Gram positive cocci to biomaterial surfaces. This adds to a recent study which showed that cranberry juice can inactivate intestinal pathogens such as *E. coli* O157:H7 (12). The effect is all the more impressive given that a regular 300 mL drink of Ocean Spray cranberry juice delivers around 30 ~40 mg of PAC, thus finding an effect with only 1 mg concentration used here, implies that an even greater effect may occur in people consuming the juice. This then correlates with the previous studies on spinal cord injured and neurogenic bladder patients where daily intake of juice reduced Gram positive and Gram negative biofilms in the bladder (9,10). The ability of PAC to inhibit and kill pathogenic bacteria could have important consequences for the care of patients at high risk of morbidity and mortality, such as those in hospitals where multi-drug resistant *S. aureus* infections are a major problem (13). The A-type proanthocyanidins responsible for the antibacterial effects are also found in plums, avocados, peanuts, curry, and cinnamon (14). If these foods were found to deliver concentrations of at least 1000 $\mu\text{g}/\text{mL}$ PAC, it seems feasible that they might provide some added protection against infection and be worth testing in hospitalized patients. Other properties, such as anti-angiogenic, antioxidant, and anti-carcinogenic properties ascribed to PACs (15), make them an attractive natural component for sick patients as well as general consumers. The one caveat to this intervention is that intake, at least of cranberry juice, would need to be monitored so as not to increase any risk of side effects such as interactions with drugs including warfarin (16) and increased risk of some kidney stone types being formed (17,18).

In terms of reducing the risk of infections associated with catheters or ureteral stents, the PACs would have to be present in excreted urine, and to date this has not been confirmed. The concentration required appears to be quite low (25 $\mu\text{g}/\text{mL}$) to reduce bacterial adhesion, but given the propensity for Gram positive cocci to adhere to these devices (19), the impact could be beneficial to many patients. The relatively rapid effect of PAC on planktonic bacteria is not only important for eradicating bacteria in suspension (such as urine) prior to their adhesion to surfaces, but also reduces the adherence of dead bacteria which by themselves can form a substrate onto which live bacteria can adhere and multiply.

The poor adherence of fimbriated *E. coli* to biomaterials has been reported previously (20). Thus while PACs can reduce adhesion of these organisms to epithelial cells, they may be less likely to reduce infection risk in catheterized patients. Having stated that, it should be noted that a previous study did find that cranberry extracts could weaken *E. coli* adhesion and biofilms on glass coverslips (21). Another factor which complicated the ability to make general conclusions about patients requiring catheters, comes from the finding that fimbriae are not essential for infection in patients with neurogenic bladder disease (22), thus decreasing the potential efficacy of cranberry juice.

In summary, the potential health attributes of cranberries continue to be intriguing and their potential to reduce infectivity of Gram positive cocci, makes PACs and foods producing these compounds, worthy of further investigation.

ACKNOWLEDGEMENTS

The cooperation of Dr. Amy Howell at the Philip E. Marucci Center for Blueberry and Cranberry Research of Rutgers University, New Jersey, USA, is very much appreciated.

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(Received January 27, 2004; Accepted March 3, 2004)