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Bacterial Anti-adhesion Activity of Human Urine: PACran Capsule vs. Theracran Capsule Consumption

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Objectives:

- 1) Determine the *ex vivo* uropathogenic bacterial (P-type *E. coli*) anti-adhesion activity in human urine following consumption of two treatments in succession with a wash-out period between each treatment: 1) 500 mg PACran capsule, 2) 650 mg TheraCran capsule BID, measured over a 36-hr time frame with product consumed for two days at the beginning of the test period only.
- 2) Determine the *in vitro* bacterial anti-adhesion activity of the whole products.
- 3) Determine levels of PAC in each product.

1) Ex vivo Urine Study Methods:

Pre-Visit Subject Preparation:

Participant inclusion and exclusion criteria: 5 women and 5 men, healthy, between the ages of 25 and 60, no current urinary infections, no diabetes, or antibiotic use for 6 months .

Dietary restrictions: participants refrained from consuming all cranberry, blueberry, pomegranate, grape, chocolate and other high-flavonoid products for a 3-day wash out period prior to consuming test products and throughout testing period.

Study Design

- 3-day wash out period prior to consuming test products and throughout test period
- On urine collection days, additional fluid consumption standardized participants to 240 mL every 3 hours to avoid dilution of urine samples and allow for detection of anti-adhesion activity, if present
- On test days, products were administered in the morning
- Urine (approximately 25 ml) was collected (clean-catch) by each participant prior to product consumption (time 0) and at 3, 6, 9, 24 and 36 hrs following product consumption
- Urine was centrifuged, filtered (.45 micron filter) and immediately frozen at -20C

Urine Protocol Specifics

Background urine samples were taken from all 10 participants prior to consumption of treatment products. Treatment 1 (one 500-mg PACran capsule) was administered in the morning over a 2-day period. On the morning of day 2, following juice ingestion, urines were collected prior to product consumption, at hour 3, 6, 9, 24 and 36 and immediately frozen at -20C. After a 3-day wash-out period, treatment 2 (two 650-mg TheraCran capsules) was administered, as stated above, and urines were collected and frozen at -20C.

Thawed urines were tested full strength for bacterial anti-adhesion activity utilizing an HRBC hemagglutination assay specific for uropathogenic P-fimbriated *E. coli* according to Foo et al. (*Phytochemistry*, 2000). A 30-uL drop of each urine was incubated with 10 uL of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn HRBCs (A1, Rh+) were suspended (3%) in PBS and added separately (10-uL 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.

Anti-adhesion activity of each urine sample was scored visually based on a quantitative estimation of percent agglutination of each sample using the following scale: 0 = no anti-adhesion activity, 1 = 50% anti-adhesion activity, 2 = 100% anti-adhesion activity. A score of 2 indicates significant anti-adhesion activity in the urine, whereas a score of 1 indicates moderate activity. The detection limits of the anti-adhesion assay are not high enough to allow quantification of the activity in each urine sample via a dilution series; therefore the result is presented as either a positive or a negative for the activity of each sample. Anti-adhesion assays were repeated four times per sample and the results averaged. Controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + test material, HRBC + test material, and bacteria + HRBC.

Data were analyzed statistically using ANOVA.

2) In vitro Bacterial Anti-adhesion Activity of Whole PACran and TheraCran

TheraCran sample was suspended (60 mg/ml) in PBS, neutralized with 1 N NaOH, diluted serially (2-fold), and tested for bacterial anti-adhesion activity utilizing an HRBC hemagglutination assay specific for uropathogenic P-fimbriated *E. coli* according to Foo et al. (*Phytochemistry*, 2000). A 30-uL drop of each dilution was incubated with 10 uL of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn HRBCs (A1, Rh+) were suspended (3%) in PBS and added separately (10-uL 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. The concentration at which hemagglutination activity was suppressed by 50% (minimum inhibitory concentration – MIC) was recorded as an indicator of the strength of the bacterial anti-adhesion activity. Anti-adhesion assays were repeated three times and the results averaged. Controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + test compound, HRBC + test compound, and bacteria + HRBC.

PACran has solubility issues in polar solvents. Therefore, the sample was extracted in a mid-polar solvent in an attempt to extract a soluble sample for testing. The PACran was weighed (240 mg) and dissolved in 10 mL of 70% acetone. The supernatant was dried and suspended in 2 mL of PBS. A two-fold dilution series was done and the MIC was recorded as above.

3) Extraction and Quantification of Proanthocyanidin Levels in Powders

The proanthocyanidins (PACs) were extracted from PACran and TheraCran powders using the gravimetric method according to Howell et al. (*Phytochemistry*, 2005). Reverse phase (C18) followed by adsorption chromatography (Sephadex LH-20) were used to fractionate and isolate the total PACs effective at preventing P-type *E. coli* bacterial adhesion. An aqueous sample

extract was loaded onto a C18 column, washed with water, then 15% methanol to elute off sugars and acids, followed by acidified methanol to elute the PACs. The PAC sample was dried, reconstituted in 50% ethanol and loaded onto a Sephadex LH-20 column. The flavonols and anthocyanins were eluted with 50% ethanol, followed by 70% acetone to recover the PAC fraction. The PAC elution was lyophilized and weighed to quantify the PACs.

Results and Discussion:

1) Ex vivo Urine Study

No anti-adhesion activity was detected in urines prior to product consumption. Urinary pH averaged 6.5, eliminating a bacteriostatic effect.

PACran vs TheraCran: Summing all observed anti-adhesion activity recorded for all participants over every time period yielded 29 out of a possible 120 for TheraCran, and 33/120 for PACran. The differences between the products were not statistically significant. By time period, the post-TheraCran urinary activity was significantly greater ($p < 0.05$) at the 6-hr time period than the activity for PACran, whereas at 36 hours PACran was significantly greater than TheraCran (Fig. 1). This suggests that TheraCran has a more rapid and substantial effect in the first 6 hours, which it maintains at 9 hours, but diminishes thereafter. The PACran activity appears to slowly increase over time and reaches peak activity at about 24 hours. The PACs in PACran may be higher molecular weight than the PACs in TheraCran, which could explain the differences in the pharmacokinetic patterns. Further research is needed to determine what activity levels at each time period correspond to a biologically relevant decrease in urinary tract infections. The overall data for all participants at each time period is presented in Fig. 2. Women responded similarly to each product, as did men (Fig. 3).

2) In vitro Bacterial Anti-adhesion Activity of Whole PACran and TheraCran

<u>Sample ID</u>	<u>Bioactivity (<i>in vitro</i>) Threshold (mg/mL)</u>
TheraCran	3.5
PACran	60

The activity of TheraCran powder was significantly greater than the PACran activity. This may be due to solubility issues of PACran in polar buffers used for the bioassay. It is unclear how the solubility may be affecting absorption and metabolism of the PACs in PACran.

3) Extraction and Quantification of Proanthocyanidin Levels in Powders

<u>Sample ID</u>	<u>PAC levels (mg/g)</u>
TheraCran	25.4
PACran	4

Again, it is probably that solubility issues of PACran inhibited the extractability of the PACs from the whole powder. The PACs in PACran may be highly complexed with cell wall material, making it more difficult to extract the PACs. The TheraCran powder delivers 33 mg of PAC per daily dose, which is close to the 36 mg level in a serving of Cranberry Juice Cocktail that has been shown to be effective in clinical trials.

Overall summary: TheraCran has significantly higher levels of PACs and higher *in vitro* bioactivity than PACran. There was no significant difference in overall *ex vivo* urinary bioactivity between TheraCran and PACran. TheraCran had a significantly higher spike of activity in the first 6 hours following ingestion, whereas PACran slowly increased in activity over 24 hours and then began to drop off. Additional work needs to be done to determine the level of activity needed at each time period to prevent UTIs.

Figure 1 – Comparison of all observed urinary anti-adhesion activity recorded per time period by all 10 participants.

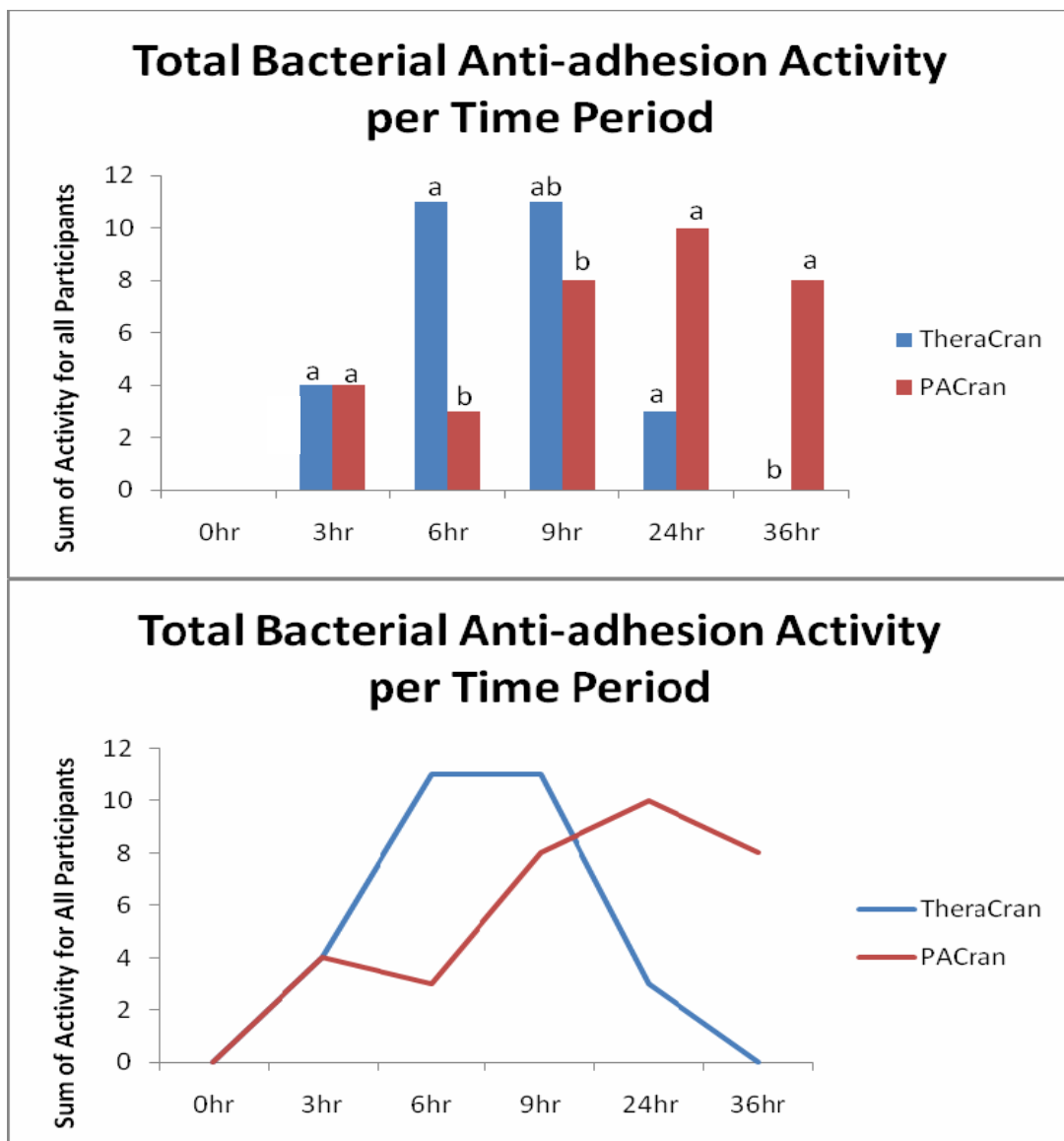


Figure 2 – Comparison of observed urinary anti-adhesion activity for each participant over each time period.

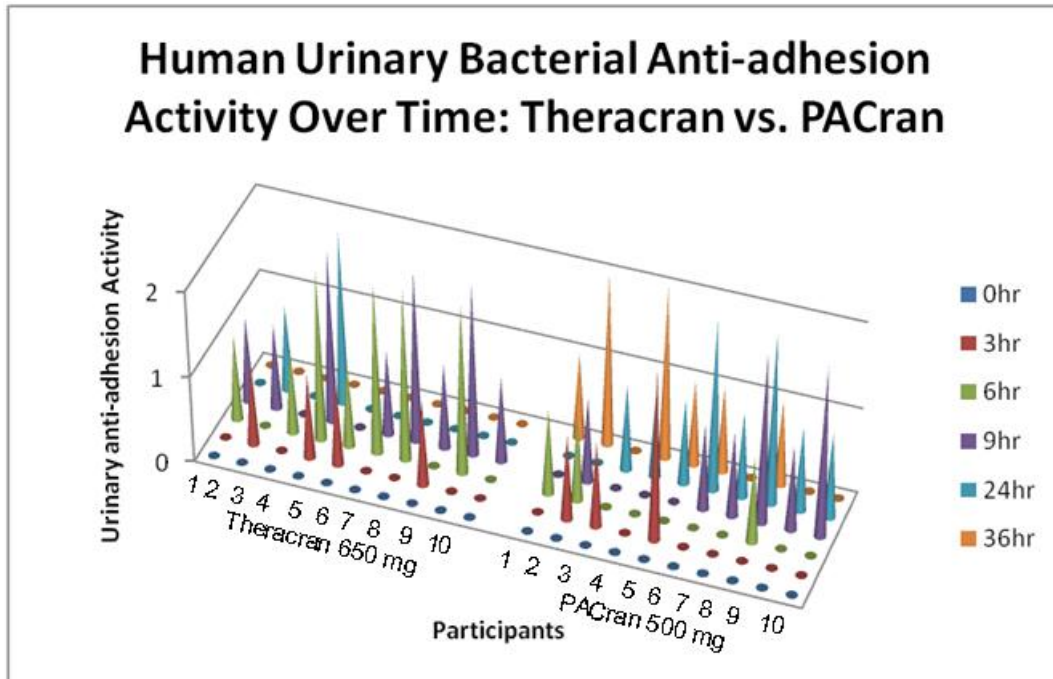
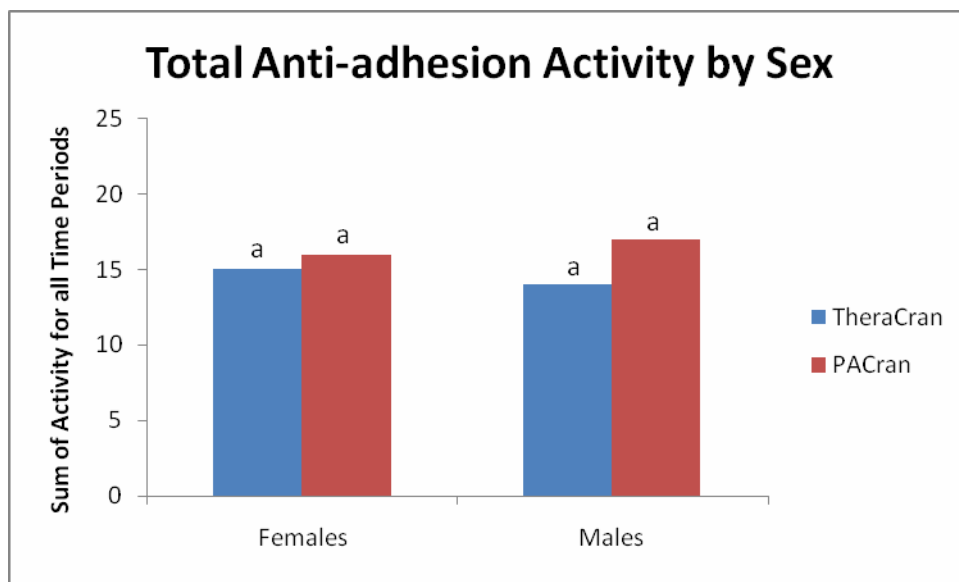


Figure 3 – Total observed urinary anti-adhesion activity recorded for women and men for each product.



Raw Data Set:

rep	treat	0hr	3hr	6hr	9hr	24hr	36hr
1F	TheraCran	0	0	1	1	0	0
2F	TheraCran	0	1	0	1	1	0
3F	TheraCran	0	0	1	0	0	0
4F	TheraCran	0	1	2	2	2	0
5F	TheraCran	0	1	1	0	0	0
6M	TheraCran	0	0	2	1	0	0
7M	TheraCran	0	0	2	2	0	0
8M	TheraCran	0	1	0	1	0	0
9M	TheraCran	0	0	2	2	0	0
10M	TheraCran	0	0	0	1	0	0
1F	PACran	0	0	1	0	0	1
2F	PACran	0	1	1	1	0	2
3F	PACran	0	1	0	0	1	0
4F	PACran	0	0	0	0	1	2
5F	PACran	0	2	0	0	1	1
6M	PACran	0	0	0	1	2	1
7M	PACran	0	0	0	1	1	0
8M	PACran	0	0	1	2	2	1
9M	PACran	0	0	0	1	1	0
10M	PACran	0	0	0	2	1	0

Participants 1-5 are women

Participants 6-10 are men