Journal of Functional Foods 18 (2015) 608–616

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/jff

Phenolic acid content and antiadherence activity in the urine of patients treated with cranberry syrup (*Vaccinium macrocarpon*) vs. trimethoprim for recurrent urinary tract infection

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ARTICLE INFO

Article history:
Received 11 May 2015
Received in revised form 19 July 2015
Accepted 17 August 2015
Available online

Keywords:
Phenolic acid
Cranberry
Urinary tract infection
Biofilm
Surface hydrophobicity
E. coli

ABSTRACT

The effectiveness of cranberry in the treatment of urinary tract infection (UTI) has been associated with its polyphenol content, particularly proanthocyanidins (PACs) and the inhibition of adherence of *Escherichia coli* to the uroepithelium. This paper describes a controlled, double blind, clinical trial of children aged over one month with recurrent urinary tract infection. The study aims were to evaluate the safety and efficacy of cranberry syrup in children and to investigate the relationship between the excretion of phenolic acids in urine with the antiadherent activity of cranberry syrup.

In the study population, cranberry syrup was found to be similar to trimethoprim, with a rate of UTI (reinfection) of 26% (95% CI 12–41). The administration of cranberry syrup was associated with high levels of hydroxycinnamic and hydroxybenzoic acids in urine; in both cases these molecules present activity in the biofilm inhibition or reduction of surface hydrophobicity of *E. coli* (Clinical Trials Registry ISRCTN16968287).

1. Introduction

The evidence currently available supports the long-term use of antibiotics in subtherapeutic doses to control recurrent urinary tract infection (UTI) (Jepson, Williams, & Craig, 2012). However, a major problem in this respect is the resulting increase in bacterial resistance to antibiotics and the selection of multiresistant bacterial flora (Gupta, 2003). Trimethoprim, which is frequently used in the treatment of UTI, is eliminated

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http://dx.doi.org/10.1016/j.jff.2015.08.009
1756-4646/

primarily by the kidney after glomerular filtration and tubular secretion. Concentrations of trimethoprim are considerably higher in urine than in the blood. After oral administration, 50–60% of trimethoprim is excreted in the urine within 24 hours, approximately 80% of this being unmetabolised trimethoprim. Trimethoprim blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase. This binding is much stronger for the bacterial enzyme than for the corresponding mammalian enzyme. Thus, trimethoprim selectively interferes with the bacterial biosynthesis of nucleic acids and proteins. Most studies have focused on changes occurring *in vitro*, leaving unresolved certain questions regarding the clinical significance *in vivo*. Various authors have used a murine urinary tract infection model to explore the potential impact of low-dose antibiotics on pathogenesis. It has been shown, using *in vitro* models, that subinhibitory antibiotics prime uropathogens for adherence and invasion (Goneau et al., 2015; Uberos, Augustin, Liebana, Molina, & Munoz-Hoyos, 2001).

Other studies have reported the usefulness of cranberry in treating recurrent UTI, in both adults and children (Beerepoot et al., 2011; Uberos et al., 2012). Cranberry extract contains at least two chemical constituents that have been associated with antiadherent properties: on the one hand, fructose inhibits the adherence of the type 1 fimbriae of some bacteria to the epithelium (Zafriri, Ofek, Adar, Pocino, & Sharon, 1989); on the other, proanthocyanidins (PACs) inhibit the adherence of P fimbriated *Escherichia coli* to the urothelium (Foo, Lu, Howell, & Vorsa, 2000; Miyazaki et al., 2002). Studies have established that after the ingestion of cranberry, the concentrations of PACs in urine are at the limits of detectability (Iswaldi et al., 2013), although some metabolites derived from PACs, such as phenolic acids, do present varying concentrations of PACs in the urine (Fernandez-Puentes et al., 2015).

The recurrence of UTI after pyelonephritis can reach 20% (Garin et al., 2006). It has been recommended (American Academy of Pediatrics, Committee on Quality Improvement Subcommittee on Urinary Tract Infection, 1999) that all cases of UTI in children aged two months to two years, after antibiotic treatment for a period of 7–14 days, should be treated with antibiotics until imaging studies are completed. However, later evidence (Garin et al., 2006; Mathews et al., 2009) suggests that antibiotic prophylaxis does not reduce renal scarring, even in patients with high-grade reflux, and so its usefulness has been questioned.

Other studies (Craig et al, 2009) have shown that paediatric patients with recurrent UTI who are treated with trimethoprim-sulphamethoxazole at low doses experienced a 6% reduction in the risk of UTI compared with a placebo group (95% CI: 1–11).

In the present study, we consider the antiadherent properties of the urine of patients included in a clinical trial and treated with cranberry syrup vs. trimethoprim for the treatment of recurrent UTI, through an analysis of phenolic acid excretion in urine and its association with: 1) the alteration of surface hydrophobicity and, 2) with the inhibition of biofilm formation by *E. coli*.

2. Materials and methods

The study is based on a Phase III randomised double-blind intervention, with two branches: one with cranberry extract in a 3% glucose syrup and the other with trimethoprim. Over a period of two years, children aged 1 month to 13 years, treated at the nephrology and urology departments of our hospital, were recruited to the study population. The maximum follow up of each such patient was one year. The trial was approved by the local ethics committee and in all cases written informed consent was obtained from the parents of the patients.

The inclusion criteria applied were a history of recurrent UTI (more than two episodes of infection in the last six months) with or without vesicoureteral reflux of any grade. Exclusion criteria were the coexistence of UTI with other infectious diseases, the coexistence of metabolic diseases, chronic renal failure, kidney stones, liver failure, allergy or intolerance to any of the components of cranberry syrup or trimethoprim, the coexistence of blood dyscrasias and the manifest wish of the person legally responsible not to participate in the study.

The cranberry extract was provided by a branded supplier and administered in the form of a 3% glucose syrup (Urell®, Pharmatoka, Labs, Rueil-Maldmaison, France). The trimethoprim was administered in a mask suspension of glucose syrup at a concentration of 8 mg/mL and CC-1000-WS (E-120) (Lab. CHR-Hansen S.L.; Tres Cantos, Madrid, Spain) had been added, at a concentration of 0.1% (in no case exceeding the permitted level of 100 ppm carmine).

The experimental group received an evening dose of 0.2 mL/kg of cranberry syrup (equivalent to 5.6 mg/kg of cranberry extract). The control group received an evening dose of 0.2 mL/kg (1.6 mg/kg of trimethoprim). The product was administered before dinner, and if vomiting occurred within 30 minutes, administration of the full dose was repeated. At all times, the intention to treat principle was maintained.

In the follow up process, periodic reviews were performed every two months or when requested by the patient if symptoms so required. The patients were instructed to perform a urine culture in their health centres if fever, urinary symptoms, vomiting or weight loss occurred. UTI was confirmed by a pathological urinary sediment (more than 20 leucocytes per field) and urine culture (>100,000 CFU/mL) from a clean-catch urine sample or a sample collected into a urine collection bag, after chlorhexidine antisepsis of the urethral meatus, or more than 10,000 CFU/mL if the urine was obtained after catheterisation.

The urine culture was always performed before starting any antibiotic treatment. The following data were recorded: the number assigned to the intervention, the clinical and analytical results of each scheduled review and the results of the periodic urine cultures. The effectiveness of an intervention was assessed in terms of the time the patient remained in the study without presenting a recurrent UTI. When such an infection took place, causing the patient to leave the study, the time of its occurrence was recorded.

To calculate the sample size required, we assumed a 20% risk of recurrence of UTI during the first year of antibiotic treatment (Garin et al., 2006). The main hypothesis tested in this study is that treatment with cranberry syrup is equivalent to

treatment with trimethoprim. An equivalence limit of ±10% was assumed. The alpha error was assumed to be 5% and the power of the study, 80%. With these data, a sample size of 109 patients per group was found to be required.

2.1. Controlling for bias

False positive or false negative results may be obtained from the urine cultures, and the possibility of information bias from this source must be taken into account. Thus, a negative urine culture may be obtained from a patient who actually has a UTI if they received antibiotic treatment before the urine sample was collected. Therefore, all patients were advised of the need to obtain a urine culture, preferably by catheterisation, in the event of any type of fever and before starting any antibiotic treatment. On the other hand, a positive urine culture may be obtained by an inadequate technique in collecting the urine, producing contamination of the sample. A urine culture that is positive to more than one micro-organism, together with normal urinary sediment, is considered contaminated and the test should be repeated.

2.2. Urine sample preparation and cranberry syrup characterisation

For infants, the first morning urine was collected in a collecting bag. For older children, it was collected by spontaneous urination, in both cases after thorough washing of the genital area. The urine was centrifuged at 4000 *g* for 10 minutes, and the supernatant was extracted and stored at –40 °C until required for processing.

The cranberry syrup was characterised at the Department of Analytical Chemistry, Faculty of Sciences, University of Granada (Iswaldi et al., 2012), and provided as a dispersion of 2.8% cranberry extract (Gykacran®) in glucose syrup. The composition of the cranberry syrup, with respect to its different polyphenol fractions, has been published previously (Iswaldi et al., 2012). The composition of the cranberry syrup used in our study is shown in Table 1.

2.3. Determination of phenolic acids and antiadherent properties in urine

Levels of phenolic acid in urine were determined in urine samples from 57 randomly-chosen patients from the ISRCTN16968287 clinical assay, at the Institute of Public Health and Clinical Nutrition, Kuopio, Finland. Of these 57 patients, 32 received trimethoprim and 25 patients received cranberry syrup.

The following phenolic acids, with diverse benzoic, acetic, propionic and cinnamic acid derivatives, were analysed; *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, gallic, 3,4-dihydroxyphenylacetic, *m*-hydroxyphenylacetic, homovanillic, 3,4-dihydroxyphenylpropionic, *m*-hydroxyphenylpropionic, dihydroferulic, dihydroisoferulic, caffeic, ferulic, sinapinic, isoferulic, *p*-coumaric and *m*-coumaric. These compounds cover a wide range of metabolites and dietary phenolic acids; they constitute a fragmentation product of procyanidins (Déprez et al., 2000; Gonthier, Donovan et al., 2003; Wiese et al., 2015) and were measured to evaluate fragmentation into other phenolic metabolites. Analysis of phenolic acids also provides information about the fragmentation of flavonols and catechins (Rios et al., 2003; Russell & Duthie, 2011; Ward, Croft, Puddey, & Hodgson, 2004). The phenolic acids in the cranberry syrup samples were first hydrolysed with enzymes (obtained from Helix Pomatia; BioSepra, France) and then with sodium hydroxide.

2.3.1. Bacterial strains, media and growth conditions

Four strains of uropathogenic *E. coli* (787, 472, 695 and 629) were used, obtained from patients with acute pyelonephritis, together with two strains of *E. coli* obtained from the Spanish Type Culture Collection (CECT): CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis) and CECT 417 (SupE44(am), mutant tRNA).

2.3.2. Inhibiting the formation of biofilm (Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000)

The strains were incubated at 37 °C for 24 hours in glass tubes with 2.5 mL of tryptic soy broth (TSB) culture medium. A volume of 0.5 mL of each such culture and 50 mcl of the urine to be studied was then introduced into a 2 mL Eppendorf tube. The negative control used was an Eppendorf tube with an equal amount of TSB without inoculum; the positive control was 0.5 mL of the bacterial suspension in a similar tube together with 50 μL of phosphate buffer saline (PBS). After 24 hour incubation, the content of each tube was carefully aspirated with a pipette and the tubes were washed three times with 1 mL of PBS. The tubes were air dried and 200 μL of 99% methanol were added as a fixative. It was left to react for 15 minutes, after which the excess was discarded and the tubes allowed to air dry. As a colorant, 0.2 mL of Hucker's crystal violet solution (2% dye content) was added and left to act for 5 minutes. The supernatant was then discarded by immersing the tubes in a trough containing distilled water. After air drying, 1 mL of 33% acetic acid was added. The optical density (OD) of the suspension in each tube was measured at 570 nm, and the zero measure was adjusted with 33% acetic acid. The results obtained are expressed as (Δ biofilm), which is the ratio between the OD of the strain incubated with the urine sample and the OD of the strain after incubation with an equal volume of PBS.

2.3.3. Determining surface hydrophobicity by analysis of salt aggregation

The biological activity on surface hydrophobicity by the polyphenol content of the urine was analysed by the aggregation technique with ammonium sulphate, following the technique described by Lindahl, Faris, Wadstrom, and Hjerten (1981). The *E. coli* culture was performed in 2 mL of TSB medium, as specified above, to enhance the expression of P-type fimbriae, after washing three times with PBS and centrifuging at 4000 *g* for 10 minutes. It was then resuspended in 0.002 M sodium phosphate (OD 1 at 540 nm). Ten microlitres of the urine sample were taken and incubated for 30 minutes at room temperature in a rotary shaker with 100 μL of the bacterial suspension of the selected strains, in PBS. Several solutions of ammonium sulphate were then prepared, at osmolarities of 0.2–4.0 M, in sodium phosphate, 0.002 M. Then, 10 μL of the bacterial suspension were mixed on a slide with an equal volume of ammonium sulphate solution. The presence of aggregation

Table 1 – Phenolic compounds in cranberry syrup, expressed in μg/mL of syrup (n = 5).

Class/phenolic compounds	μg/mL cranberry syrup
Phenolic acid derivatives	
Coumaroylglucose/*p*-Coumaric acid 4-*O*-glucoside	113.39 ± 4.11
1-*O*-Sinapoyl-*β*-D-glucose/4-*O*-*β*-D-Glucosyl-sinapate	21.44 ± 0.73
Caffeoyl glucose	39.27 ± 1.24
Chlorogenic acid	38.70 ± 1.07
p-Coumaroyl-glucose	80.35 ± 2.31
Canthoside A	3.23 ± 0.08
2-Hydroxybenzoic acid	98.79 ± 2.95
Gallic acid 3-*O*-gallate	116.72 ± 3.46
Flavonols	
Myricetin 3-*O*-galactoside/glucoside	251.74 ± 9.74
Dihydro Ferulic Acid 4-*O*-*β*-D-Glucuronide	47.92 ± 2.42
Myricetin 3-*O*-arabinoside	453.26 ± 17.65
Caviunin glucoside	594.61 ± 26.87
Quercetin 3-*O*-galactoside/glucoside	782.27 ± 38.59
Quercetin 3-*O*-xylopiranoside	136.27 ± 4.74
Quercetin 3-*O*-arabinopyranoside	132.78 ± 4.34
Quercetin 3-*O*-arabinofuranoside	241.06 ± 8.63
Quercetin 3-*O*-rhamnoside	261.02 ± 8.46
Myricetin	229.57 ± 7.59
Methoxyquercetin 3-*O*-galactoside	nq
Kaempferol 3-*O*-*β*-D-(6″-*p*-hydroxybenzoyl)-galactopyranoside	61.40 ± 2.78
Quercetin	606.54 ± 25.63
Syringetin	49.45 ± 1.67
Proanthocyanidins	
Procyanidin B-type	405.68 ± 14.78
(+)-Catechin	748.63 ± 30.86
Procyanidin A2 isomer 1	728.05 ± 29.78
Procyanidin B-type	431.28 ± 18.21
Procyanidin A2 isomer 2	713.98 ± 25.77
Procyanidin A2 isomer 3	1288.74 ± 99.42
Prodelphinidin B4	419.97 ± 14.74
Isoflavonoids	
Biochanin A-7-*O*-glucoside	nq
Coumarins	
7-Hydroxycoumarin	493.83 ± 17.54
Coumarin	349.80 ± 12.43
Scopoletin	899.99 ± 39.64
Anthocyanin	
Petunidin	19.17 ± 5.81
Total	10,858.91 ± 389.62

nq, not quantified.

was observed after one minute at room temperature. Note was taken of the lowest concentration of ammonium sulphate that produced visible aggregation on the slide after gentle manual rotation for 30 seconds. Aggregation with 4M solutions was interpreted as 0% hydrophobicity, and aggregation with 0.2M solutions was interpreted as 95% hydrophobicity. The results obtained are expressed as Δ hydrophobicity, which is the ratio of the hydrophobicity of the strain incubated with the urine sample and the hydrophobicity of the strain after incubation with an equal volume of PBS (Table 2).

2.4. *Statistical analysis*

The statistical analysis of patient follow-up took the form of a Kaplan–Meier survival analysis, in which the appearance of the study event (UTI) concluded the follow-up period. Cases of voluntary withdrawal from the study or of departure from causes other than UTI were considered to be censored for the rest of the monitoring period. The Kaplan–Meier curves for the experimental intervention and for the standard treatment were compared. Another comparison was made of the urinary excretion of polyphenols in each of the study groups, using a t-test

Table 2 – Mean adherence (biofilm formation) and hydrophobicity of the strains of *E. coli* studied.

E. coli strains	Δ adh	% Hydrophobicity
4076	1.74	90
471	0.97	95
787	1.38	95
695	1.30	80
417	1.05	95
629	1.13	85
Total	1.26 (DS 0.27)	90 (DS 6.3)

for independent samples. Linear and multiple linear regression analyses were performed for the excretion of the different phenolic acids in urine and for the antiadherent properties of the urine samples.

The clinical analysis of non-inferiority was conducted by considering the difference in UTI prevalence between the two study branches (d), taking into account the standard error in each case (SEt), in accordance with the following expression:

$$SEt = \sqrt{\frac{SE\,(cranberry)}{n1} + \frac{SE\,(trimethoprim)}{n2}};$$

where SE is the standard error for each treatment branch. The equivalence limits were calculated as ± 0.10 (10%) $\rightarrow \delta_L = -0.10$, $\delta_U = 0.10$; for a value $z_\alpha = 1.96$.

The test of non-inferiority for trimethoprim vs. blueberry was analysed using the following unilateral hypothesis:

$$H1_L = Pr_{cranberry} - Pr_{trimethoprim} > \delta_L \rightarrow H0_L$$
$$= Pr_{cranberry} - Pr_{trimethoprim} \leq \delta_L$$

which is evidenced by $z_L = \frac{d - \delta L}{SEt} \geq Z\alpha$

$$H1U = Pr_{cranberry} - Pr_{trimethoprim} > \delta_U \rightarrow H0_U$$
$$= Pr_{cranberry} - Pr_{trimethoprim} \geq \delta_U$$

which is evidenced by $z_L = \frac{d - \delta L}{SEt} \leq -Z\alpha$

3. Results

3.1. *Effectiveness of cranberry syrup in preventing the recurrence of urinary infection*

The following subjects were recruited to the study: 85 children under 1 year of age, 53 of whom were treated with trimethoprim and 32 with cranberry syrup; 107 children over 1 year of age, 64 of whom were treated with trimethoprim and 43 with cranberry syrup.

Among the children under 1 year of age, the rate of UTI associated with trimethoprim treatment was 19% (95% CI 4–35) in the boys and 43% (95% CI 18–68) in the girls. In the children under 1 year of age, the rate of UTI associated with cranberry syrup treatment was 46% (95% CI 23–70) in the boys and 17% (95% CI 0–38) in the girls. Irrespective of sex, the rate of UTI in the patients receiving trimethoprim was 28% (95% CI 13–42) and 35% (95% CI 17–52) in those receiving cranberry syrup.

Overall, the children over 1 year of age had a rate of UTI of 35% (95% CI 21–50) among those receiving trimethoprim and of 26% (95% CI 12–41) among those receiving cranberry syrup. By gender, the boys who received trimethoprim had an infection rate of 33% (95% CI 1.8–65) compared with 8% (95% CI 0–26) for those receiving cranberry syrup.

The CONSORT diagram (Fig. 1) shows the adverse events detected in the children (both groups: those aged under 1 year and those aged over 1 year) during the follow-up period. There were notably few adverse reactions, and none at all among the children aged under 1 year.

The recurrent episodes of UTI were caused by *E. coli* in 60% of the cases, with no significant differences between the two treatment groups. 33.3% of the patients receiving trimethoprim presented urine cultures that were positive for multi-resistant bacteria, versus 22.9% of those receiving cranberry syrup.

For the sample as a whole, $Z_L = 2.04 > Z_\alpha$ and $Z_U = -1.65 > -Z_\alpha$; therefore, we conclude that cranberry syrup treatment is similar to trimethoprim treatment. In infants aged under 1 year, we obtained $Z_L = 0.69 < Z_\alpha$ and $Z_U = -1.9 > -Z_\alpha$; accordingly, we find that in these infants, the cranberry syrup treatment, at the dose administered, is inferior to trimethoprim.

3.2. *Urinary excretion of phenolic acids, with respect to biofilm formation and surface hydrophobicity of* E. coli

Table 3 shows the concentrations of phenolic acids recorded for the first morning urine samples from patients receiving trimethoprim or cranberry syrup treatment (a single evening dose) for UTI. There were significant differences in the urinary levels of ferulic acid and of *m*-hydroxybenzoic acid.

Simple linear regression analysis revealed a significant association between surface hydrophobicity and *m*-hydroxybenzoic acid, and multiple regression analysis enabled us to derive a predictive model with $R^2 = 0.45$ ($F = 8.3$; $p < 0.001$) that included *m*-hydroxybenzoic acid, 3,4-dihydroxyphenylpropionic acid and homovanillic acid ($y = 0.74 - 0.05 \times m$-hydroxybenzoic acid $- 0.03 \times$ 3,4-dihydroxyphenylpropionic acid $+ 0.003 \times$ homovanillic acid).

According to the simple linear regression analysis, the inhibition of biofilm formation by *E. coli* is associated with the concentration of isoferulic acid in urine. Multiple regression analysis produced a predictive model that incorporated isoferulic acid and ferulic acid with $R^2 = 0.26$ ($F = 5.49$; $p < 0.01$) ($y = 0.65 + 0.05 \times$ isoferulic acid $- 0.01 \times$ ferulic acid).

4. Discussion

Our study confirms that cranberry syrup is similar to trimethoprim treatment for recurrent UTI in children and infants, although in the latter case its effectiveness is considered inferior to that of trimethoprim at the doses used in our study. Furthermore, we find that cranberry syrup treatment at this dose is safe in infants and children.

In 1984, Sobota (1984) observed that cranberry interferes with the adhesion of P-fimbriated *E. coli* to the epithelium, and showed that this could be one of the main mechanisms accounting for the non-adherent effect of cranberry. Two mechanisms may be involved in this process: a) the fructose present in many fruit juices, including cranberry, may also inhibit the adhesion of *E. coli* mediated by type 1 fimbriae (Zafriri et al., 1989); b) the adherence mediated by P fimbriae has been associated with the lectin-specific α-Gal(1-4)β-Gal present in the urothelium, which is not inhibited by the addition of fructose (Di Martino et al., 2006; Salminen et al., 2007). Foo et al. (2000) identified trimeric proanthocyanidins and type A dimeric procyanidins as responsible for the antiadherent effect of

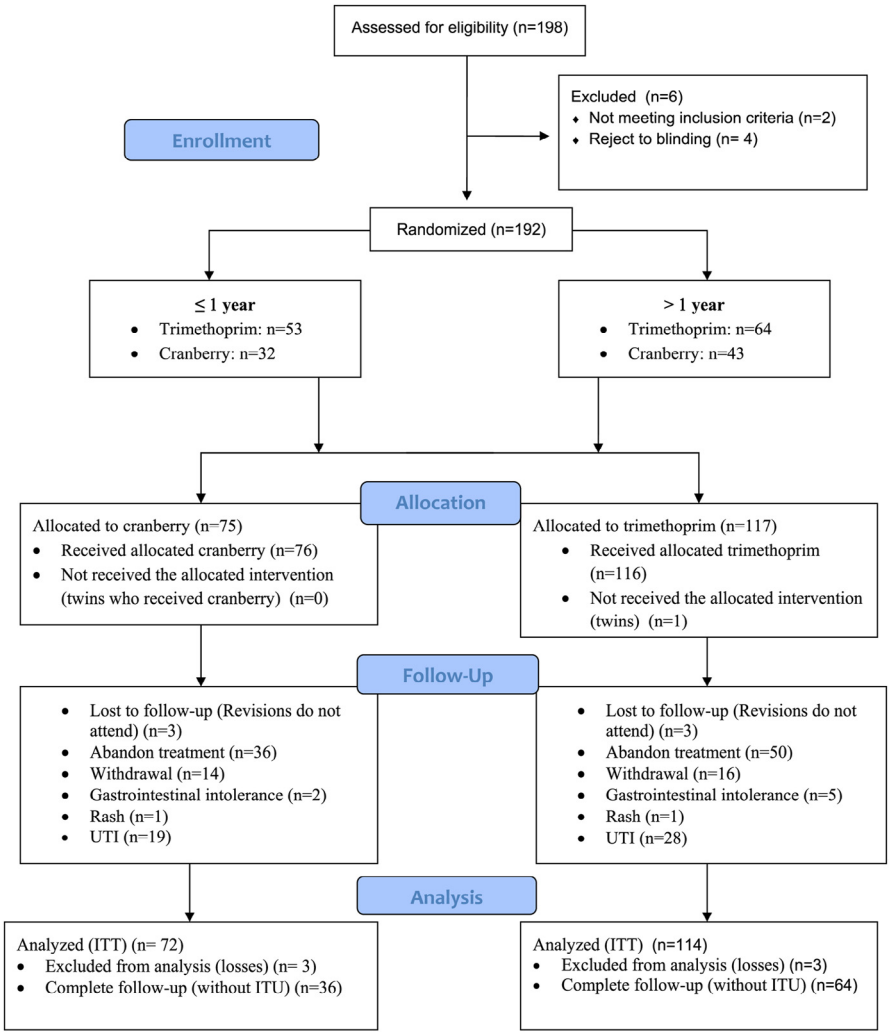

Fig. 1 – Flow diagram of the clinical trial.

Table 3 – Phenolic acid content (mean (SD)) in the first morning urination of patients receiving nocturnal trimethoprim or cranberry treatment. Duplicate measurements. We observed levels of ferulic, isoferulic and *m*-hydroxybenzoic acids in patients taking cranberry.

(μMol/g creatinine)	Cranberry	Trimethoprim
Protocatechuic acid	3.6 (2.9)	4.1 (4.1)
Syringic acid	1.3 (2.6)	0.5 (0.7)
Caffeic acid	1.2 (2.6)	0.6 (2.4)
Ferulic acid	13.5(16.1)*	7.8 (9.8)
Sinapinic acid	0.7 (4.2)	0.02 (0.2)
p-Coumaric acid	1.6 (4.4)	0.5 (1.2)
Isoferulic acid	4.3 (5.4)**	2.6 (4.0)
Dihydroferulic acid	4.9 (9.6)	4.7 (7.4)
3,4-dihydroxyphenylpropionic acid	2.3 (3.4)	3.3 (5.2)
m-Hydroxyphenylpropionic acid	4.4 (16.2)	1.9 (4.0)
p-Hydroxyphenylpropionic acid	0.9 (5.1)	1.2 (9.3)
Homovanillic acid	94.9 (50)	77.8 (38)
3,4-Dihydroxyphenylacetic acid	23.7 (16.8)	25.0 (18)
m-Hydroxyphenylacetil acid	36.5 (50)	24.4 (31)
p-Hydroxyphenylacetic acid	334 (355)	308 (328)
Vanillic acid	141 (154)	185 (440)
m-Hydroxybenzoic acid	4.47 (3.1)*	1.98 (4.0)
p-Hydroxybenzoic acid	34 (24)	33 (43)
Total phenolic acid	696 (598)	685 (668)

* $P < 0.05$.
** $P < 0.01$.

Table 4 – Linear regression analysis – B coefficient and the standard error (SE) – of the variability of surface hydrophobicity and the inhibition of biofilm formation by *E. coli* in the urine samples and the corresponding phenolic acid content. In patients receiving cranberry the increment of urine levels of caffeic, 3,4-dihydroxy-phenylpropionic and *m*-hydroxybenzoic acids associated with decrease in surface hydrophobicity of *E. coli*.

	Δ Hydrophobicity			Δ Biofilm inhibition		
	Cranberry	Trimethoprim	Total	Cranberry	Trimethoprim	Total
Protocatechuic acid	0.004 (0.13)	−0.012 (0.02)	−0.09 (0.01)	−0.016 (0.013)	−0.011 (0.024)	−0.01 (0.01)
Syringic acid	0.009 (0.15)	0.01 (0.12)	0.002 (0.002)	0.005 (0.015)	−0.06 (0.106)	−0.004 (0.02)
Caffeic acid	−0.07* (0.02)	0.019 (0.01)	0.009 (0.01)	0.023 (0.033)	−0.003 (0.02)	0.0001 (0.01)
Ferulic acid	0.001 (0.003)	0.002 (0.009)	0.003 (0.004)	0.003 (0.003)	−0.001 (0.01)	0.001 (0.004)
Sinapinic acid	0.002 (0.008)	0.000 (0.000)	0.007 (0.01)	0.003 (0.007)	0.000 (0.000)	0.001 (0.01)
p-Coumaric acid	−0.07 (0.04)	0.106 (0.116)	0.01 (0.06)	0.042 (0.05)	0.21 (0.11)	0.09 (0.05)
Isoferulic acid	−0.01 (0.01)	0.044* (0.02)	0.01 (0.01)	0.012 (0.01)	0.05* (0.02)	0.02* (0.01)
Dihydroferulic acid	0.000 (0.000)	−0.007 (0.011)	−0.01 (0.007)	−0.002 (0.01)	−0.005 (0.008)	−0.003 (0.006)
3,4-dihydroxy-phenylpropionic acid	−0.03* (0.01)	−0.01 (0.01)	−0.01 (0.008)	0.005 (0.018)	−0.007 (0.012)	−0.003 (0.008)
m-Hydroxy-phenylpropionic acid	0.001 (0.01)	−0.006 (0.01)	−0.007 (0.01)	−0.006 (0.012)	0.014 (0.014)	0.01 (0.009)
p-Hydroxy-phenylpropionic acid	0.04 (0.11)	0.000 (0.000)	0.11 (0.18)	0.118 (0.109)	0.000 (0.000)	0.08 (0.17)
Homovanillic acid	0.002 (0.002)	0.001 (0.002)	0.001 (0.001)	−0.002 (0.002)	0.002 (0.002)	0.001 (0.001)
3,4-Dihydroxy-phenylacetic acid	0.005 (0.007)	0.001 (0.003)	−0.001 (0.003)	−0.012 (0.006)	0.0001 (0.003)	0.0001 (0.003)
m-Hydroxy-phenylacetic acid	0.001 (0.001)	−0.001 (0.003)	0.0001 (0.001)	0.0001 (0.002)	0.004 (0.003)	0.002 (0.002)
p-Hydroxyphenyl-acetic acid	0.0001 (0.0001)	0.001 (0.001)	0.0001 (0.001)	0.0001 (0.0001)	0.0001 (0.0001)	0.0001 (0.0001)
Vanillic acid	0.0001 (0.0001)	0.001 (0.001)	0.0001 (0.0001)	−0.001 (0.0001)	0.001 (0.001)	0.0001 (0.0001)
m-Hydroxybenzoic acid	−0.043* (0.02)	−0.045* (0.02)	−0.05** (0.01)	−0.08 (0.01)	−0.018 (0.023)	−0.01 (0.18)
p-Hydroxybenzoic acid	0.004 (0.003)	−0.002 (0.002)	−0.002 (0.002)	0.0001 (0.003)	−0.001 (0.002)	0.0001 (0.002)
Total phenolic acid	0.0001 (0.0001)	0.0001 (0.001)	0.001 (0.001)	0.0001 (0.0001)	0.0001 (0.0001)	0.0001 (0.0001)

* $P < 0.05$.
** $P < 0.01$.

cranberry. These polyphenolic flavonols are not present in other extracts rich in polyphenols, such as green tea or chocolate. The cranberry syrup used in our test contained 22% type A proanthocyanidins, an aspect of interest in the comparison of our results with those reported elsewhere, given the considerable variability in the concentrations of proanthocyanidin measured in different berry extracts (Latti, Kainulainen, Hayirlioglu-Ayaz, Ayaz, & Riihinen, 2009) (Foo et al., 2000). The systematic review by Jepson et al. (2012) of ten studies, with a total of 1049 patients, concluded that cranberry compared to placebo/control significantly reduced the incidence of UTI during 12 month follow-up (RR 0.65; 95% CI 0.46–0.90). Cranberry more effectively reduced the incidence of recurrent UTI in women than in men and older women, and in patients requiring catheterisation. Kontiokari et al. (2001) demonstrated the beneficial effect of cranberry in preventing UTI in women, reducing the absolute risk of UTI compared to placebo treatment. Ferrara et al. (2009), in a controlled clinical trial of children aged over three years, found that cranberry, versus placebo, prevented the recurrence of symptomatic urinary infections. Our study is a controlled, randomised, double-blind clinical trial that demonstrates the safety of cranberry in infants and children. Howell et al. (2010) found that the antiadherent activity of cranberry was greater when the concentration of the proanthocyanidin administered with the cranberry exceeded 18 mg. In our own study, in the absence of previous references on the use of cranberry in infants, we decided to extrapolate from the adult dose, and adopted a dose of 0.2 mL of cranberry syrup per kg body weight. Our results show that cranberry syrup treatment is not inferior to trimethoprim when a dose ensuring more than 18 mg PACs is provided, which is the dose per kg body weight that would be administered to an infant aged 1 year. This circumstance would explain the poor performance obtained for the infants in our study. Since our preliminary results were obtained, the majority of commercial presentations of cranberry have increased the PAC content to levels above the 36 mg considered in our study.

The antiadherent effect of PACs on bacterial adherence has been demonstrated *in vitro* by various authors (Di Martino et al., 2006; Miyazaki et al., 2002; Uberos et al., 2012). However, *in vivo*, PACs are rapidly metabolised in the liver and intestine and the metabolites are eliminated in the urine (Bravo, 1998; Xiao et al., 2009). In a clinical trial of adults, doses 3 times higher than those used in this study were administered, and the level of PACs in the urine could not be quantified (Iswaldi et al., 2013). In total, 32 metabolites were tentatively identified, including methylated and glucuronide conjugated forms. Among these, free phenolic acid derivatives (coumaroyl hexose, dihydroxybenzoic acid, caffeoyl glucose and dihydroferulic acid 4-O-β-D-glucuronide), flavonols (methoxyquercetin 3-O-galactoside, myricetin and quercetin) and one coumarin (scopoletin) were detected in the human urine. The highest concentration of these metabolites in urine is reached at 4 hours after the consumption of cranberry. Further studies are currently in progress, seeking to extend our knowledge of the bioavailability of cranberry-derived polyphenols and to obtain new insights into active metabolites (Iswaldi et al., 2013).

Sublethal doses of antibiotics, including trimethoprim, seem to effectively reduce the surface hydrophobicity of *E. coli* (*in vitro*). This effect has been observed by various authors (Fu, Hassett, & Cohen, 1989; Uberos et al., 2001), and seems to be related to the alteration of cell wall protein synthesis by the antibiotic.

Phenolic acids are considered to be products of the fragmentation of the procyanidins, flavonols and catechins present in the diet or in cranberry treatment (Déprez et al., 2000; Gonthier, Verny, Besson, Remesy, & Scalbert, 2003). We

recorded levels of ferulic acid and of *p*-coumaric acid that were slightly higher, or at the limit of statistical significance, in the group receiving cranberry. No significant differences were observed in the other phenolic acids studied.

Hydroxybenzoic acid (gallic acid) and hydroxycinnamic acid (ferulic acid) have shown antibacterial properties on *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes* (Borges, Ferreira, Saavedra, & Simoes, 2013). Our data corroborate the relation between the increased presence of hydroxybenzoic acid in urine and a decrease in the surface hydrophobicity of *E. coli* (Table 4). Other authors (Sadowska, Paszkiewicz, Podsedek, Redzynia, & Rozalska, 2014) have shown that extracts of *Vaccinium myrtillus* with a high total content of phenolic compounds, especially hydroxycinnamic acid, hydroxybenzoic acid and flavonols, enhance the biocidal potential of vancomycin and linezolid against staphylococci.

Isoferulic acid is a methylated metabolite of chlorogenic acid (CGA). Phenolic compounds such as CGAs are abundantly present in certain foods. CGAs are formed by the esterification of hydroxycinnamic acids, such as caffeic, ferulic and coumaric. Other metabolites of microbial origin, namely, *m*-coumaric acid and derivatives of phenylpropionic, benzoic and hippuric acids, represented the major phenolic compounds in both urine and plasma (Gonthier, Donovan et al., 2003). In accordance with the findings of Borges et al. (2013), our results show that the presence of isoferulic acid in urine is associated with decreased biofilm formation by *E. coli*.

In view of the rapid metabolism *in vivo* of the phenolic compounds in the cranberry, which are subsequently eliminated in the urine, we believe that some metabolites of cranberry, such as hydroxybenzoic acid or hydroxycinnamic acid, may also be responsible for the antibacterial effects described *in vivo*.

Acknowledgement

This study was funded by Institute of Health Carlos III (Spain) (PI07-0274).

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