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ORIGINAL PAPER

Inhibition of adherence of multi-drug resistant *E. coli* by proanthocyanidin

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Abstract Proanthocyanidin is commonly used for inhibiting urinary tract infection (UTI) of sensitive strains of *Escherichia coli*. The aim of this study was to investigate the effect of proanthocyanidin on adherence of uropathogenic multi-drug resistant *E. coli* to uroepithelial cells, which has not yet been investigated so far. Extracts of the purified proanthocyanidin were prepared from dried cranberry juice. Purity and structural assignment of proanthocyanidin was assessed using high performance liquid chromatography and ^{13}C nuclear magnetic resonance spectroscopy, respectively. Subsequently, its affect on multi-drug resistant bacteria as well as quantification of anti-adherence bioactivity on human vaginal and bladder epithelial cells was appraised. Inhibition of adherence to an extent of about 70% with multi-drug resistant *E. coli* strains was observed on uroepithelial cell. The anti-adherence bioactivity of the proanthocyanidin was detected at concentrations of 10–50 μg/ml with significant bacteriuria. Probable proanthocyanidin through A-type linkages either combines to P-fimbriae of bacterial cells or modifies the structural entity of P-fimbriae and inhibits bacterial adherence to uroepithelial cells. The proanthocyanidin exhibited anti-adherence property with multi-drug resistant strains of uropathogenic P-fimbriated *E. coli* with in vitro study. Hence proanthocyanidin may be considered as an inhibitory agent for multi-drug resistant strains of *E. coli* adherence to uroepithelial cells.

Keywords Proanthocyanidin · Bacterial adherence · Multi-drug resistance *E. coli*

Abbreviations

NMR	Nuclear magnetic resonance
HPLC	High performance liquid chromatography
RBCs	Red blood cells
E. coli	*Escherichia coli*
VECs	Vaginal epithelial cells
BECs	Bladder epithelial cells
MF	McFarland
CFA	Colonization factor agar
AMC	Amoxicillin–Clavulanic Acid
UTI	Urinary tract infection
PBS	Phosphate buffer saline solution

Introduction

Urinary tract infection (UTI) indicates the presence of microorganisms in the kidney, prostate, urinary bladder, or genitourinary system (urethra, vagina, etc.) and results in significant morbidity, particularly in women and children [1]. It affects about eight million people per year in the

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United States alone [2]. Some women are more prone to get recurrent infections which not only frustrate the patients but also contribute to the increase of bacterial resistance to antibiotics. UTIs are commonly caused by predominant (80%) uropathogenic gram-negative *Escherichia coli* [3]. *E. coli* adherence to uroepithelial cells, a prerequisite for development of UTIs, is facilitated by proteinaceous macromolecules: Type-1 and P-fimbriae [4, 5].

A cornerstone of prevention of UTI is becoming very important in the light of increasing bacterial resistance to antibiotics, making the challenges of treating UTIs more difficult. Microorganisms are not only becoming more and more intricate because of their etiological heterogeneity but also quite cumbersome, expensive, and inaccessible to all to get successful treatment. So there is a demand for the exploration of alternative remedies to prevent and treat UTIs. Thus, the attraction to the natural herbal remedies appears because it is easy to get, inexpensive, and substantially effective.

The literature reveals that the consumption of cranberry (*Vaccinium macrocarpon*) juice helps prevent UTI [6–11]; it has been used by Native Americans for more than 200 years [12]. Several theories regarding the effect of cranberry juice have been proposed by different studies [6]. Results suggest that the effect is not due to change in the physical properties (like acidic pH) of urine but to specific compounds in cranberries that inhibit the adherence of *E. coli* to uroepithelial cells. Cranberries contain fructose, which has been implicated in the inhibition of the adherence of *E. coli* with type-I fimbriae [7]. Another compound—proanthocyanidin—identified in the cranberry juice is found responsible and considered to be the main active ingredient for inhibiting P-fimbriated *E. coli* adherence to uroepithelial cells [8]. The inhibition of adherence activity of this compound has been observed not only to sensitive but also for trimethoprim–sulfamethoxazole-resistant uropathogens [9] and to asymptomatic bacteriuria in patients with an ileal enterocystoplasty [11]. Furthermore, it has not been evaluated and investigated with multi-drug resistant uropathogens, which remains unexplored to date. Therefore, the present study was designed to address this issue and evaluate proanthocyanidin's effects on both sensitive and multi-drug resistant bacteria—either it has the same effect on both the population or the multi-drug resistant bacteria have some mechanism to surpass its inhibitory effect—on human vaginal epithelial and bladder epithelial cells, and to human red blood cells (RBCs). These observations will lend support to the existing information for sensitive bacteria and expand the beneficial effects of proanthocyanidin for multi-drug resistant bacteria towards the prevention of UTIs and improvement of their clinical management.

Materials and methods

Extraction and isolation of proanthocyanidin

Extracts of purified proanthocyanidin were prepared as previously described [10] from the dried powder of cranberry juice (PYCNOGEN, USA). The purity of proanthocyanidin extracts was evaluated through performance liquid chromatography (HPLC) with respect to the standard compound obtained from Sigma–Aldrich, St. Louis, MO 63178, USA.

The HPLC analysis was performed with Shimadzu SPD-10 A. equipped with UV/Vis detector 200 nm and auto-sampler. The proanthocyanidin isolated from the powdered cranberry juice (1 mg) dissolved in 0.5 ml methanol was subjected to solid-phase extraction using a Bond-Elute C_{18} cartridge (Baker Bond column). The column was preconditioned prior to loading the sample with repeated elutions of 2 ml of acetonitrile–water (3:1 v/v). After loading the sample, the column was washed with 2 ml of HPLC grade water. The HPLC system was operated isocratically at 0.4 ml/min flow rate for the mobile phase acetonitrile–water (3:1 v/v) at room temperature. Detector response was recorded on a strip chart recorder. Similarly, standard proanthocyanidin (1 mg) obtained from Fluka, USA, dissolved in 0.5 ml of methanol was applied on a C_{18} cartridge; for comparison, analysis was performed under similar conditions explained for isolated proanthocyanidin.

The structural confirmation of the isolated compound was determined by nuclear magnetic resonance (NMR) spectroscopy. ^{13}C NMR spectrum of proanthocyanidin sample was recorded on a Bruker Avance NMR spectrometer at operating frequency of 100.62 MHz for ^{13}C. The spectrum was obtained using one pulse sequence, with proton decoupling using WALTZ-16 composite pulses. Typical parameters used for ^{13}C NMR experiment were the following: spectral width 24,000 Hz, data points 32 K, flip angle 90°, relaxation delay 2 s, spectrum size 32 K points, and line broadening 8 Hz.

Bioactivity testing of proanthocyanidin

Selection of bacterial strains

Strains of *E. coli* isolated from urine samples of patients with clinically suspected UTI were tested with Gal–Gal coated latex beads for the presence of P-fimbriae, using test kits obtained from Kavivitrum, Stockholm, Sweden. Only P-fimbriae containing *E. coli* strains were used for the study. Antibiotic susceptibilities were tested according to Clinical Laboratory Standard Institute (CLSI) recommendations [13] against the following drugs: Ampicillin,

Amoxicillin–Clavulanic Acid (AMC), Cefazolin, Cefuroxime, Trimethoprim–Sulphamethaxazole, Ciprofloxacin, Gentamicin, Amikacin, and Nitrofurantoin. Twenty P-fimbriated *E. coli* strains; ten sensitive to all drugs and ten multi-drug resistant (strains resistant to three or more antimicrobials are considered as multi-drug resistant); were selected. These P-fimbriated *E. coli* strains were sub-cultured over casamino acids yeast extract agar (also known as colonization factor agar, CFA) and grown overnight at 37°C to enhance production of P-fimbriae. Strains were harvested, washed once, and suspended in 5 ml phosphate-buffered saline (PBS) to make stock suspensions of 9×10^8 bacteria/ml (obtained by matching with McFarland Number 3). Two-fold serial dilutions of the above prepared stock suspensions were made and the second dilution corresponding to 9×10^6 bacteria/ml was taken for subsequent adherence assays on uroepithelial cells.

Hemagglutination assay

The anti-adhesion bioactivity of the proanthocyanidin was tested by measuring the ability of the fractions to suppress agglutination of human RBCs (A1, Rh+). Stock solutions of bacteria corresponding to concentration 9×10^8 bacteria/ml were used for the study. 50 ml stock solution of proanthocyanidin, contains proanthocyanidin concentration varying from 150 μg/50 μl to 750 μg/50 μl were prepared. A 10 μl drop of each solution was incubated with 10 μl of bacterial suspension on a 96-well micro-dilution polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn human RBCs (blood group A, Rh+) were suspended (3%) in PBS and added separately (10 μl drops) to the above prepared test suspension (proanthocyanidin + bacterial suspension) and to 10 μl of proanthocyanidin-free bacterial suspension, which was diluted with 10 μl of normal saline in place of proanthocyanidin. Suspension were incubated for half an hour on a rotary shaker at 37°C and observed microscopically for hemagglutination inhibition.

Cell culture

Approval for the study was obtained by institutional ethics committee. Written informed consent was obtained from all participants. The two types of cells-one vaginal epithelial cells (VECs) and second, bladder epithelial cells (BECs) were used for this study. VECs and BECs were obtained from the clinical discarded human tissue samples following standard tissue culture techniques [14–16]. Briefly, culture media, antibiotics, antimycotics, and other supplement media were obtained from Sigma–Aldrich and Gibco (Carlsbad, CA 92008, USA). After transferring the tissue samples from the operation room to the tissue culture lab, the epithelial mucosa was washed with PBS solution to remove blood cells. Uroepithelial cells (VECs and BECs) were carefully scraped from the underlying muscle layer with a bone curette under sterile conditions. The isolated uroepithelial cells were transferred in serum-free Ham's F-12 medium along with essential supplements [14–16]. Consequently, uroepithelial cells were washed, seeded in culture plates, and incubated at 37°C in a humidified atmosphere of 5% CO_2 [14–16].

Bacterial adherence assays on uroepithelial cells

Stock solutions of proanthocyanidin with concentrations varying from 20 to 100 μg/ml were prepared. 500 μl of each proanthocyanidin stock solution was added to 500 μl of bacterial solutions containing 9×10^6 bacteria/ml (log 2 dilution of the original bacterial suspension), so that the final concentrations of proanthocyanidin varying from 10 to 50 μg/ml were obtained. The mixtures of proanthocyanidin solution and bacterial suspension were incubated at 37°C for 30 min. Consequently, 200 μl of each mixture were inoculated into the cell culture flask containing 1.5×10^6 uroepithelial cells (VECs and BECs, separately). After another 30 min of incubation, uroepithelial cells were washed with 50 ml PBS. Bacterial cells in washed PBS were counted by standard pour plate method [17–19].

Adhesion assays using proanthocyanidin-free bacterial suspension were also done and used as controls. For this 500 μl of the bacterial suspension and 500 μl of normal saline (in place of proanthocyanidin solution) were mixed and inoculated in a cell culture flask. After 30 min of incubation, uroepithelial cells were washed; bacterial cells in the washed PBS were counted, as described previously [17–19].

The average percentages of inhibition of *E. coli* were measured after repeating the five times of the complete protocol of bacterial adherence assays on uroepithelial cells with VECs and BECs, separately.

Statistical methods

Data were analyzed using Graph Pad INSTAT 3.0 software. A probability p value of less than 0.05 was taken to indicate statistical significance. *E. coli* adherence to uroepithelial cells were plotted against PAC concentrations from 0 to 50 μg/ml. The mean inhibition of adherence of *E. coli* with different concentration of proanthocyanidin was summarized and presented in Fig. 4a, b.

Results

The retention times (R_t) for the isolated fraction and pure standard sample of proanthocyanidin were compared. The observed retention time of the extracted and standard compound was within standard error. The observed R_t was 8.99 and 9.07 s for laboratory-isolated and standard proanthocyanidin, respectively, (Fig. 1a, b). ^{13}C NMR spectra reveal the structural entity of laboratory-isolated proanthocyanidin (Fig. 1c).

Effect of proanthocyanidin on hemagglutination by P-fimbriated *E. coli*

The hemagglutination activities of all the strains (sensitive as well as resistant) of P-fimbriated *E. coli* were very high against human RBCs. The inhibitory effect of proanthocyanidin on hemagglutination by P-fimbriated bacteria, observed after 30 min of incubation, was dependent on the quantity of the proanthocyanidin. As the quantity of proanthocyanidin increases concomitantly, hemagglutinating activity of P-fimbriated *E. coli* decreases (Fig. 2a–l). The observation suggests that in the absence of proanthocyanidin as well as in presence of lower quantities of proanthocyanidin, the hemagglutination was highly positive (4+ and 3+) but with higher concentrations of proanthocyanidin the hemagglutination was weakly positive or negative (1+ or −).

Effect of proanthocyanidin on adherence of *E. coli* to uroepithelial cells

Inhibition of adherence of the P-fimbriated *E. coli* strains on uroepithelial cells in the presence of proanthocyanidin was observed for both sensitive and multi-drug resistant bacteria (Fig. 3a–l). Inhibition of adherence of *E. coli* strains to uroepithelial cells was found to be proportional to the increase in quantity of proanthocyanidin, numbers of uroepithelial and bacterial cells being constant. All the sensitive as well as multi-drug resistant *E. coli* strains have shown almost similar patterns of inhibition of adherence on uroepithelial cells. The inhibition of adherence of sensitive and multi-drug resistant strains of *E. coli* to uroepithelial cells was observed up to 70% with assays of 0 to 50 μg/ml

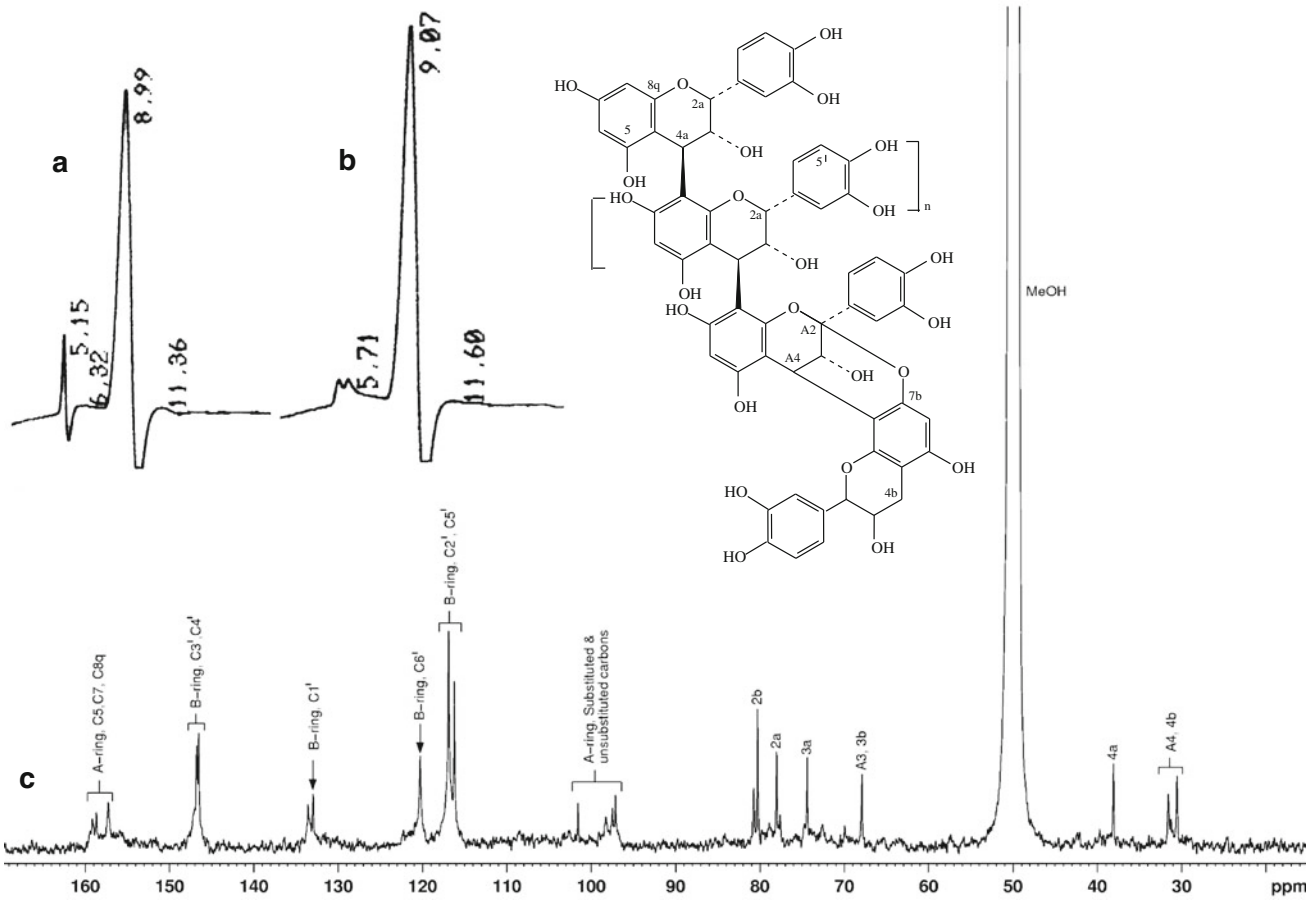

Fig. 1 HPLC chromatogram and NMR spectrum of proanthocyanidin (**a**) Extracted from dried cranberry juice (**b**) standard compound obtained from Fluka, USA (**c**) ^{13}C NMR spectrum of proanthocyanidin extracted from dried cranberry juice along with their chemical structure

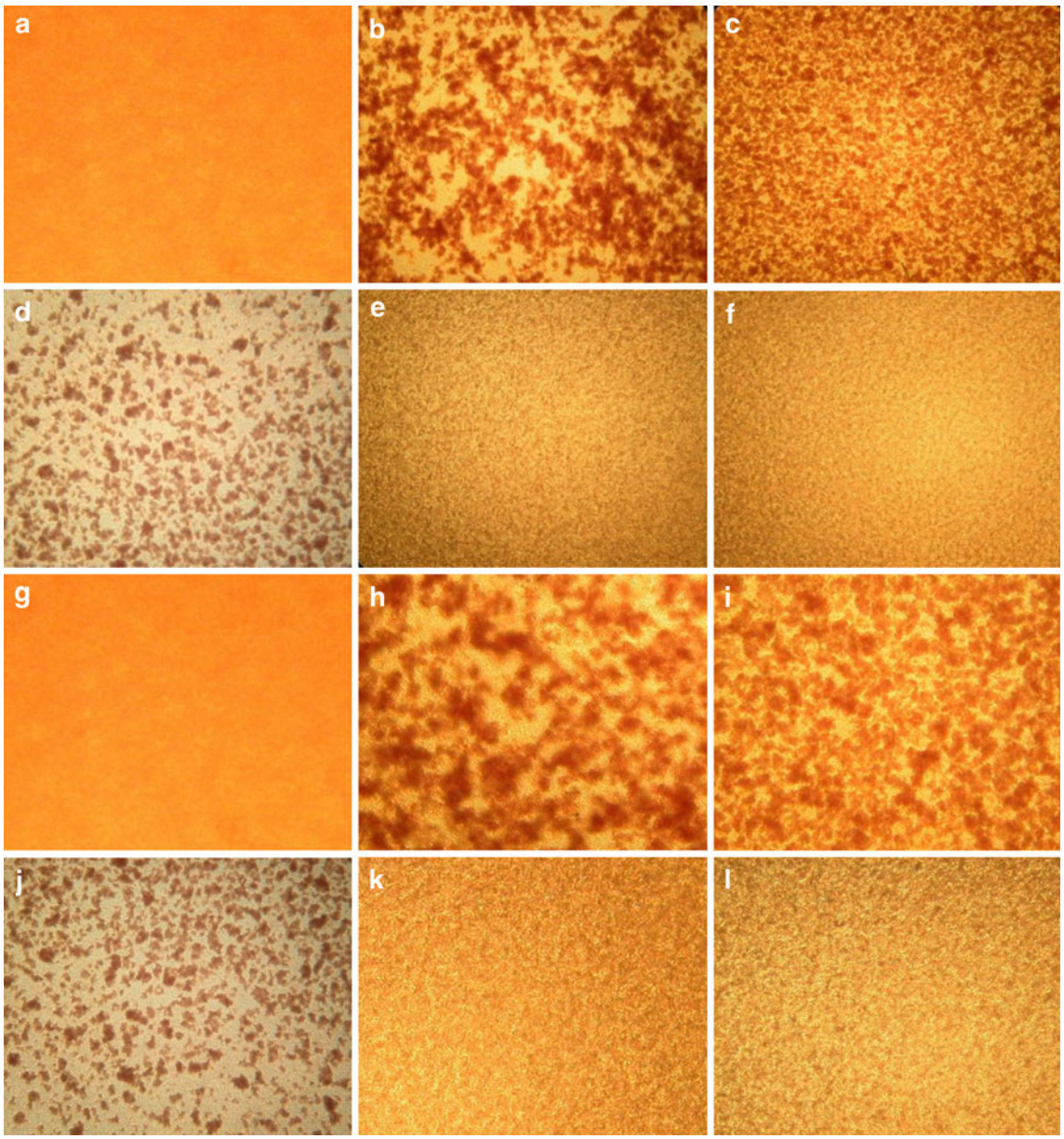

Fig. 2 **a** Control human RBCs and **b–f** anti-hemagglutination assays of sensitive *E. coli* and human RBCs with increasing concentration of proanthocyanidin; **b** 10, **c** 20, **d** 30, **e** 40, **f** 50 μg/ml. **g** control human RBCs and **h–l** anti-hemagglutination assays of multi-drug resistant *E. coli* and human RBCs with increasing concentration of proanthocyanidin; **h** 10, **i** 20, **j** 30, **k** 40, **l** 50 μg/ml

of proanthocyanidin and significant bacteriuria (Fig. 4a). With 10 μg/ml of proanthocyanidin (2.0×10^5 bacteria adhere on cells) versus 0 μg/ml (2.5×10^5 bacteria adhere on cells) exhibited statistically significant ($p < 0.001$, *t* test) inhibition of adherence. Similarly other experiments also exhibited significant inhibition of adherence for subsequent concentration of proanthocyanidin (Fig. 4a, b). The results of the adherence of *E. coli* assays demonstrated with increasing doses of proanthocyanidin from 0 to 50 μg/ml (Fig. 4b). With 50 μg/ml of proanthocyanidin (0.9×10^5 bacteria adhere on cells) versus 0 μg/ml (2.5×10^5 bacteria adhere on cells) exhibited statistically significant ($p < 0.0001$, *t* test) inhibition of adherence. The mean results of inhibition of adherence with various amount of proanthocyanidin are presented in (Fig. 4a, b).

Discussion

This study investigated the ability of proanthocyanidin to inhibit the adherence of sensitive and multi-drug resistant strains of uropathogenic *E. coli* using human uroepithelial cells. The finding of the present study demonstrates that proanthocyanidin is capable of inhibiting the adherence to

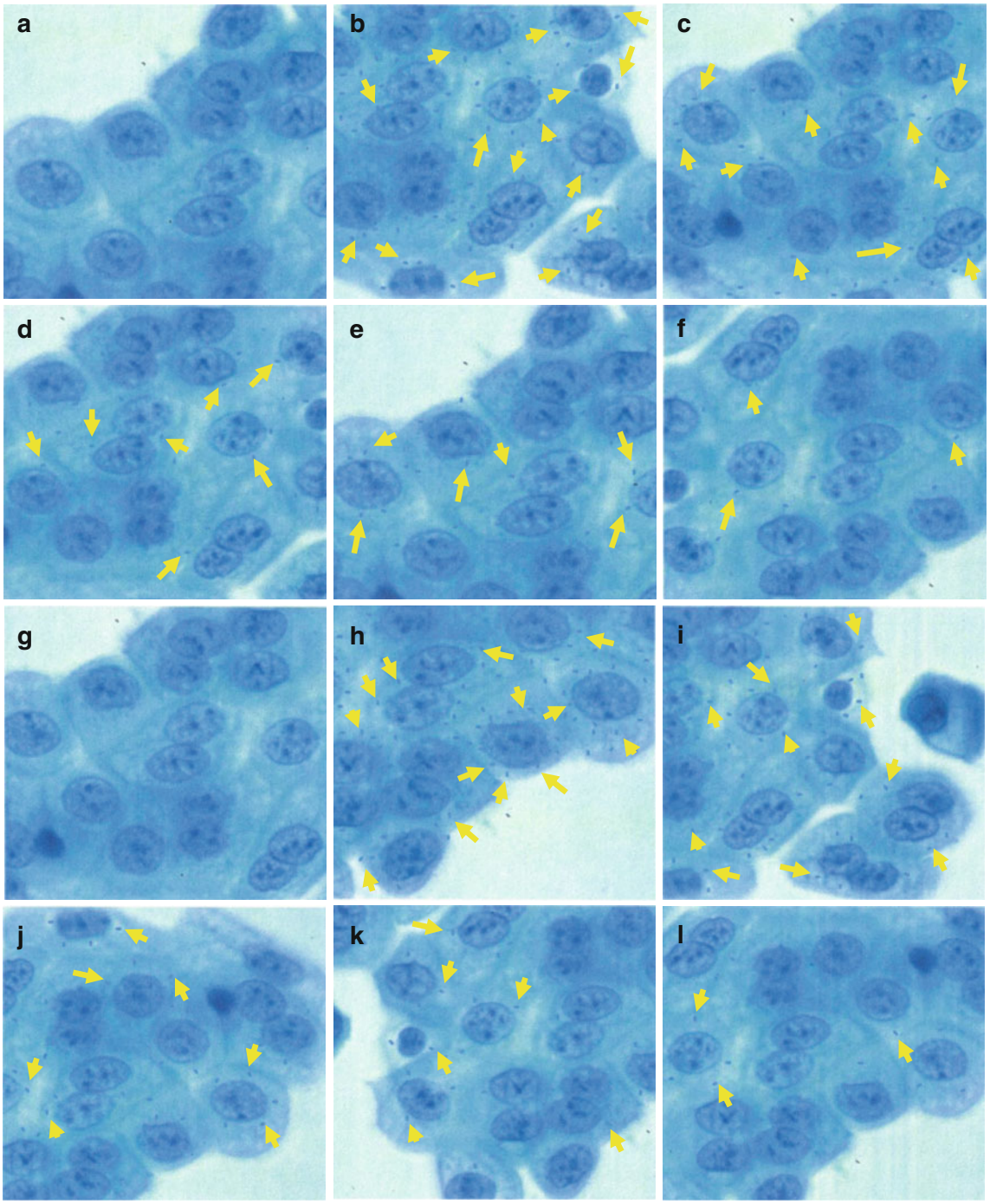

Fig. 3 **a** Control uroepithelial cells and **b–f** inhibition of adherence of *E. coli* (sensitive strain) on uroepithelial cells with increasing concentration of proanthocyanidin; **b**10, **c** 20, **d** 30, **e** 40, **f** 50 μg/ml. *Yellow color arrow* representing the adherence of *E. coli* to uroepithelial cells. **g** Control uroepithelial cells and **h–l** inhibition of adherence of *E. coli* (multi-drug resistant strain) on uroepithelial cells with increasing concentration of proanthocyanidin; **h** 10, **i** 20, **j** 30, **k** 40, **l** 50 μg/ml. *Yellow color arrow* representing the adherence of *E. coli* to uroepithelial cells

not only sensitive but also multi-drug resistant uropathogenic *E. coli*. This inhibition process increases with dose of proanthocyanidin, 10–50 μg/ml, which extent as much as 70% inhibition with approximating significant bacteriuria (around10^5 bacteria/ml) under in vitro conditions, and agrees with prior measures of 5–75 μg of proanthocyanidin [20–22]. Possibly these amount of proanthocyanidin may be enough to inhibit the significant number of *E. coli* under in vivo conditions also; hence the anti-adhesion activity was observed in the urine within 2 h and persisted for up to

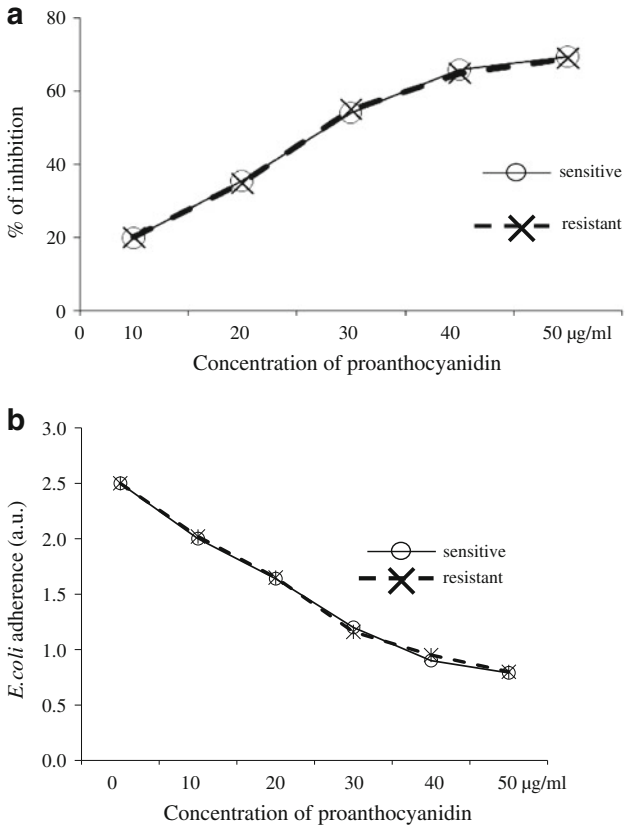

Fig. 4 **a** Percentage inhibition of both sensitive (*empty circle with light thin line*) and multi-drug resistant (*cross with dark thick dotted line*) *E. coli* as a function of concentration of proanthocyanidin. **b** Adherence of *E. coli* on uroepithelial cells in absence and presence of various concentrations of proanthocyanidin

10 h following proanthocyanidin-containing cranberry juice ingestion, previously discussed in details [11, 20–22]. The outcomes of the present study not only lend to support earlier studies of the inhibition of adherence properties of proanthocyanidin to sensitive strains of *E. coli* but also expand the use of proanthocyanidin for anti-adherence of multi-drug resistant uropathogens at least under in vitro conditions. This study may help to push forward the boundaries of use of proanthocyanidin products for preventing and treating UTIs more economically.

To date, no prior reports are available, which evaluate the effects of proanthocyanidin on the adherence of multi-drug resistant uropathogens to both human VECs and BECs. Pathogen's adherence to uroepithelial cells is a vital step for UTI instigation and subsequent colonization. So the prevention of uropathogen's adhesion to urogenital cells may serve not only in circumventing UTI but also for colonization.

Studies suggest that cranberry contains two different inhibitors; one dialyzable and other non-dialyzable component. The dialyzable or low molecular weight components of the juice (fructose, quinic acid, citric acid, malic acid and vitamin C) inhibit mannose-sensitive type 1 fimbriated bacteria in a hapten-like manner [23]. Cranberry contains one other non-dialyzable or high molecular weight moiety, proanthocyanidin, playing a major role in the inhibition of adherence of P-fimbriated *E. coli* [specific for α-D-Gal(1 → 4)-β-D-Gal] [10]. Proanthocyanidins are stable phenolic compounds that are widely distributed in nature; some of them possess antiviral, antibacterial, antiadhesive, antioxidant, and antiproliferative properties [6, 24–26]. Proanthocyanidin contains A-type linkages, can be associated with preventing adhesion of P-fimbriated *E. coli* to uroepithelial cells [21]. Different mechanisms proposed so far to explain the antimicrobial activities of proanthocyanidin include inhibition of extracellular microbial enzyme, deprivation of substrate required for microbial growth, or direct action on microbial metabolism through inhibition of oxidative phosphorylation [6, 27, 28].

Escherichia coli is the most common uropathogenic bacterium and its P-fimbriae (which bind α-D-Gal(1 → 4) β-D-Gal present on the uroepithelial cell surfaces) are thought to be most important virulence factors in causing UTI. P-fimbriae are found in 60% strains of *E. coli* causing cystitis (urinary bladder infection) and 80% strains causing pyelonephritis (kidney infection). Proanthocyanidin blocks fimbrial adhesion to uroepithelial cells, thus preventing *E. coli* from colonizing the uroepithelial cells. This blockage of fimbrial adhesion by proanthocyanidin may be due to the astringent nature of proanthocyanidin, changing the structural configuration of fimbrial proteins of the bacteria, thus inhibiting the binding of these fimbrial proteins to P-antigens on uroepithelial cells. Because it has been observed that cranberry reduces the strength of the binding between uroepithelial and uropathogens moieties by altering the conformation of surface macromolecules (equilibrium length of P-fimbriae is shortened from 148 to 48 nm, i.e., fimbrial proteins are more compressed) [29]. This alteration in P-fimbriae of surface macromolecule is felt to be an irreversible inhibition [30].

It is also possible that the microbial cell surface receptors (epitopes) are occupied by the proanthocyanidin, instead of the paratopes of surface receptors of uroepithelial cells. Possibly microorganisms secrete outside the cell, polymers [Gal (1 → 4) Gal] having high affinity for proanthocyanidin. Proanthocyanidin combines to these compounds, covering the fimbrial surfaces of bacteria and thus competitively blocks the bacterial adherence to uroepithelial cells. Another possible mechanism is that proanthocyanidin can inhibit the protein expression of P-fimbriae of *E. coli* resulting in the inhibition of bacterial adherence to cellular surfaces. Hence the proanthocyanidin effectively prevents the adherence of P-fimbriated *E. coli* and impedes the colonization on uroepithelial cells.

Conclusion

Our results suggest that the consumption of cranberry-derived proanthocyanidin offers protection against both sensitive as well as multi-drug resistant strains of P-fimbriated *E. coli*. Thus proanthocyanidin may be used for UTI prevention with pathogens that depend on P-fimbriae. The dose scenario of proanthocyanidin is still pending to be explored with large clinical trial because correlation of oral dosage and amount of proanthocyanidin observed in urine is not established yet. This study reveals the answer of the main question as to whether proanthocyanidin is able to inhibit the adherence of multi-drug resistant strains on uroepithelial cells.

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Conflict of interest None.

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