

Anti-virulence effects of aqueous pomegranate peel extract on *E. coli* urinary tract infection

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Summary. Urinary tract infections (UTIs) are among the most prevailing infectious diseases and may be classified as uncomplicated or complicated, depending upon the urinary tract anatomy and physiology. The Gram negative bacteria of *E. coli* cause 70-95% of upper and lower UTIs. Pomegranate peels (*Punica granatum* L.) are considered byproducts obtained during juice processing and characterized by the significant presence of polyphenols associated with biological properties such as antimicrobial and antioxidant agents. The aim of this study was to estimate the antimicrobial and anti-virulence effect of aqueous pomegranate peel extract against *E. coli* cultures collected from urinary cultures of the Microbiology Laboratory of Al-Bassel Hospital, in Syria, 2016. The inhibitory activity was found to be dose and pH dependent with an MIC value of 0.6 mg/ml and an MBC value of 1.2 mg/ml at the pH of the aqueous extract (3.5). The assay of adhesion carried out at MIC showed a reduction of up to 80% of the adhesion index accompanied with a reduction in motility and ornithine decarboxylation as indicated by MIO test. Results indicate that the aqueous pomegranate peel extract could be an important source of new antimicrobial compounds in order to treat *E. coli* urinary tract infections.

Key words: aqueous pomegranate peel extract, antimicrobial effect, anti-virulence effect, *E. coli*, UTI

Introduction

Urinary Tract Infection (UTI) is defined as the microbial invasion of any tissues in different parts of the urinary tract and is the second most common infectious presentation in community medical practice (1). Individual susceptibility to UTI is complex, depending on several factors such as genetic, biologic, and behavioral ones. The pathogenic bacteria can adhere, grow and resist against host defenses which will result in colonization and infection of the urinary tract. Each bacterial species has distinct virulence mechanisms that facilitate UTI (2, 3).

It has been reported in several studies that the Gram negative bacteria of *E. coli* cause 70-95% of up-

per and lower UTIs (3). The severity of the infection depends both on the virulence factors of the infecting bacteria and on the vulnerability of the host. Up to 95% of UTIs occur in an ascending beginning with bacterial colonization of the periurethral area followed by infection of the bladder and may then ascend the ureters to reach the kidneys (4). If left untreated, the infection could access the bloodstream and causes bacteremia (4).

The uropathogenic *E. coli* possess adherence factors called pili or fimbriae, which allow them to successfully initiate infections and may protect the bacteria from urinary lavage, increasing their ability to multiply and invade renal tissue (5). Flagella, an organelle responsible for bacterial motility, are

involved in the interaction of various pathogenic *E. coli* strains with epithelial cells (6). The role of flagellum-mediated motility in the rise of uropathogenic *E. coli* to the upper urinary tract and in its diffusion into the bloodstream as well as in the maintenance of persistent infection has been well established (7).

A fluoroquinolone for 7-10 days can be recommended as first-line therapy and third-generation oral cephalosporin could be an alternative (8, 9). However, it has been found that the numbers of fluoroquinolone-resistant *E. coli* have increased in some parts of the world, thus restricting their use of fluoroquinolones (8, 9).

Medicinal plants have always been a good source to find new remedies for human health problems. Recently, a wide range of these plants have been screened for antimicrobial property (10).

Pomegranate peels (*Punica granatum*) are considered wastes or byproduct obtained through juice processing (11). It is characterized by significant presence of ellagitannins and polyphenols, gallic acid and ellagic acid (12) as well as flavonoids-associated with biological properties such antimicrobial agents (13).

Various extracts prepared from pomegranate fruit peels were evaluated for their antimicrobial activity against some food-borne pathogens using several methods (14-17). It was found that 80% methanolic extract of peels was a potent inhibitor for *Listeria monocytogenes*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (14-17). Alam Khan and Haneef had shown that Ethanol extract of pomegranate peels has lowest MIC against *E. coli*, *P. aeruginosa* and *S. aureus* compared to MICs of methanolic and hot water extracts (18). The inhibitory zones of all the three extracts were greater than that of the standard antibiotic Tetracycline (18). In contrast Nuamsetti et al. found that the hot water extract of the peels was most potent against *E. coli* compared to 95% ethanol and acetone extracts (19).

The objective of this study was to explore the efficacy of using aqueous pomegranate peel extract to reduce pathogenicity of *E. coli* responsible for UTI and attempt to find a safety method to solve the problem of multi-drug resistance pathogen.

Materials and Methods

Pomegranate peel extract

Fresh pomegranates were collected from Syrian markets. They were cleaned with water and dried with a cloth. The peels were manually separated, dried for a few days in an open air shade and then powdered in a blender. The moisture content was determined by using a moisture analyzer balance.

1 g of dried and ground peel was placed in a thermostatic water bath shaker with 100 ml of DI water at 50°C for 20 min. The liquid extract was centrifuged at 2000 rpm for 10 min and the supernatant was transferred to a 100 ml flask. DI water was added to make the final volume 100 ml (20).

Microbial cultures

Cultures of *E. coli* were provided from urinary culture collections of the Microbiology Laboratory of Al-Bassel Hospital, in Syria, 2016. Then, bacteria were incubated at 37±0.1°C for 24 h by injection into Nutrient Broth. A standardized suspension of *E. coli* was prepared by suspending colonies from overnight culture in peptone to obtain 1.5x10⁸ CFU/ml.

Determination of MIC and MBC

The MIC of aqueous pomegranate peel extract was evaluated using the microdilution broth method according to National Committee for Clinical Laboratory Standards, 2003. Geometric dilutions ranging from 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mg/mL of the pomegranate extracts were prepared. The standardized suspension of *E. coli* was tested in tubes against the varying concentrations of aqueous pomegranate peel extract. The tubes were incubated for 24h at 37°C and the growth of the pathogen was detected using spectrophotometer at 600 nm. Concentration in the tube showing no turbidity was considered as MIC. Aliquots of 100 µl from each transparent tube showing no turbidity were separately cultured on Eosin Methylene Blue agar (EMB) plates. After 24 h of incubation at 37°C, the concentration of antibacterial agent in the tube that showed no bacterial growth was recorded as MBC (21).

Adhesion assay

Collection of uroepithelial cells

The *in vitro* adherence of *E. coli* to uroepithelial cells was studied according to the method of Suzanne et al. (22). Uroepithelial cells were obtained from fresh urine collected over a 24-h period from normal healthy women with no history of urinary or vaginal infections and who are not taking contraceptive or antimicrobial agents. The urine was immediately centrifuged at 4000 rpm for 15 minutes, the supernatant was discarded and the uroepithelial cells were harvested by washing the sediment three times with 5ml of phosphate buffer saline (pH 5). The number of cells was calculated by direct light microscopy and an epithelial cell count of 2×10^5 cells/ml was obtained by re-suspending a suitable number of the epithelial cell in phosphate buffer saline pH 5.

In vitro assay

One ml of bacterial suspension was mixed with one ml of epithelial cell suspension. The mixture was incubated in shaking water bath at 37°C for 3 hours. Then it was washed three times and a portion of the final cell suspension was placed on a slide, air dried, methanol fixed and stained with Giemsa stain (10%) for 30 minutes and examined under light microscopy (X100). The average number of adhering bacteria per cell was obtained from an examination of 50 cells. Each test was performed in triplicate.

E. coli was grown for 36 h at 37°C in bacteriological peptone with the addition of pomegranate peel aqueous extract, at the minimum inhibitory concentration. Then the incubated bacterial suspension was placed in contact with the cells and incubated at 37°C for 3 h. Finally, they were washed with PBS to remove any bacteria that had not adhered, the cells were then air dried, methanol fixed and stained with Giemsa stain (10%) for 30 minutes, then they were observed at the microscope (X100). The average number of adhering bacteria per cell was obtained from an examination of 50 cells. Each test was performed in triplicate.

Motility Assay

A sterile needle was used to pick a well-isolated colony of *E. coli* before and after treatment with aqueous

pomegranate peel extract at MIC and stabbed into the MIO medium to within 1 cm of the bottom of the tube. Tubes were incubated at 35°C for 18 hours.

pH effect

E. coli strains grow in a broad pH range of 4.4–10.0, with an optimum pH of 6–7 (23). In order to clarify the acidic properties influence of aqueous pomegranate peel extract (pH value 3.5) on *E. coli* growth, strains were grown in three different pH ranges. The MIC and MBC of strains was evaluated using the microdilution broth method according to National Committee for Clinical Laboratory Standards, 2003 (24).

The pH of aqueous pomegranate peel extract was adjusted to 7 using sodium carbonate and a citric acid buffer was prepared at pH 3.5. Geometric dilutions ranging from 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mg/mL of the aqueous pomegranate peel extract (pH=3.5) and the modified extract (pH=7) were prepared. The standardized suspension of *E. coli* was tested in tubes against the above varying concentrations and varying dilutions of citric acid buffer. The tubes were incubated for 24h at 37°C and the growth of the pathogen was detected using spectrophotometer at 600 nm. Concentration in the tube showing no turbidity was considered as MIC. Aliquots of 100 µl from each transparent tube showing no turbidity were separately cultured on Eosin Methylene Blue agar (EMB) plates. After 24 h of incubation at 37°C, the concentration of antibacterial agent in the tube that showed no bacterial growth was recorded as MBC (21).

Results and Discussion

Determination of MIC and MBC

In general, the extent of the inhibitory effects of the pomegranate extracts could be attributed to their polyphenol content. These compounds are very abundant in aqueous pomegranate peel extract as reported by our previous work (20) and their effects on bacterial metabolism are identified by the effect of tannins, such as punicalagin, on bacterial membrane, because they can pass through cell walls and bind to their surface which prevents their normal activity (25). Punicalagin and gallic acid also showed antibacterial efficacy against

methicillin resistant *Staphylococcus aureus* strains, *Corynebacterium*, *Streptococcus*, *Bacillus subtilis*, *Shigella*, *Salmonella*, *Escherichia* and *Vibrio* species (26, 27).

As shown in Figure 1, results indicated a significant effect of pomegranate peel extract on decreasing the bacterial growth at concentration starting from 0.2 mg/ml. MIC value for aqueous pomegranate extract was 0.6 mg/ml, whereas MBC value was 1.2 mg/ml.

In different recent studies, MIC varied from 0.19–25 mg/ml against several strains of *E. coli* (16, 28). The variation in MIC can be related to the differences in the amount of antibacterial substances (such as tannins and phenolic substances) among pomegranate cultivars and genotypes. Minor differences in laboratory techniques and the strain of *E. coli* species used in the experiments run by researchers may also be involved in the variation of the reported results.

Adhesion assay

The adhesion assay carried out on the *E. coli* treated with aqueous pomegranate peel extract at MIC showed a reduction of up to 80% of the adhesion index. The count of adhering bacteria was carried out manually, both for controls and treated strains (Figure 2). About 112.4 ± 5.7 of *E. coli* bacteria adhered on the assayed epithelial cell before treatment, while only 23.6 ± 3.7 of *E. coli* adhered to the epithelial cells after treatment with aqueous pomegranate peel extract.

These results were in accordance with different previous studies (29, 30) where aqueous pomegranate peel extract worked as an anti-adhesive because of large amounts of saponins, alkaloids, and polyphenols (31–33).

Motility assay

A positive motility test is indicated by a diffuse cloud of growth away from the line of inoculation, whereas ornithine decarboxylation is indicated by a purple color in the medium. A negative ornithine reaction produces a yellow color at the bottom of the tube.

Results indicate a reduction in *E. coli* motility in tubes treated with aqueous pomegranate peel extract accompanied with a reduction in ornithine decarboxylation as in Figure 3.

Activity of ornithine decarboxylase results in production of polyamines such as putrescine and spermi-

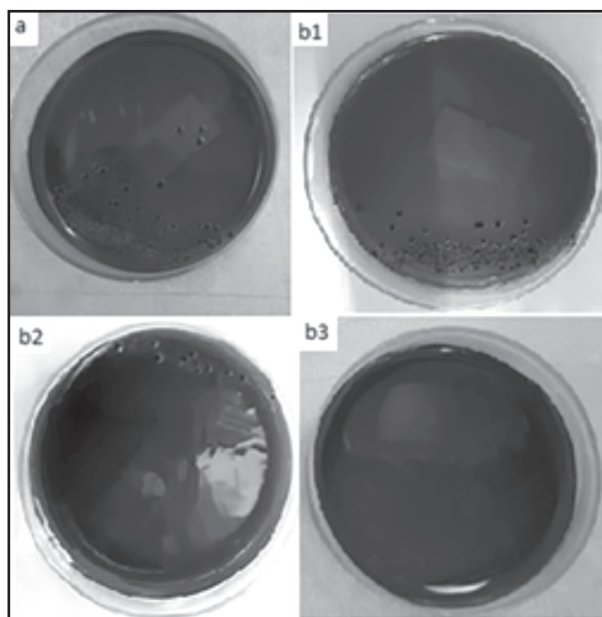


Figure 1. *E. coli* growth on EMB before (a) and after (b1, b2 and b3) treatment with aqueous pomegranate peel extract.

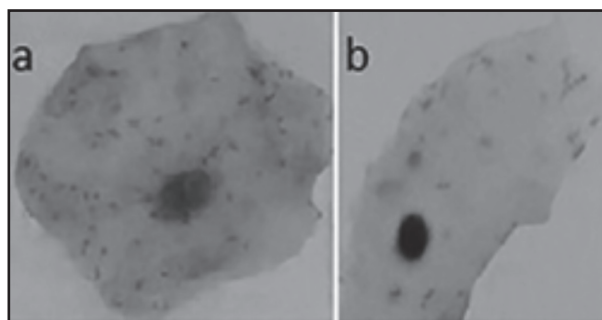


Figure 2. *E. coli* adherent to a cell on a control slide. b: *E. coli* adherent to cells after treatment with aqueous pomegranate peel extract at the concentration of 1.0 mg/ml. The lower number of adherent bacteria can be clearly seen.

dine which play an important role in biofilm formation and so in cellular adherence of *E. coli* (34).

Recently, it was shown that antibiotics such as fluoroquinolones, aminoglycosides and cephalosporins are able to induce oxidative stress, a substantial contributor to cell death by the damage of protein and DNA (35). Polyamines help *E. coli* to survive with stress conditions, such as oxidative radicals (36) and low pH (37) which eventually results in a considerable increase in cell viability, growth recovery and antibiotic resistance.

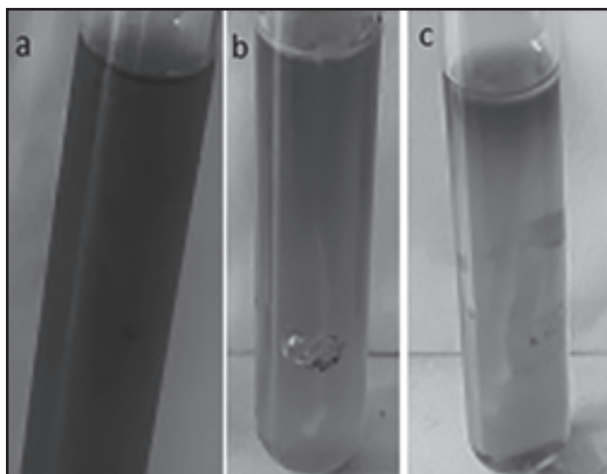


Figure 3. MIO test for the motility of *E. coli*. a: the MIO medium, b: MIO medium inoculated by *E. coli* untreated by aqueous pomegranate peel extract, c: MIO medium inoculated by *E. coli* treated by aqueous pomegranate peel extract at MIC.

Based on these results, an attractive idea is that of potentiation of antibiotic effects in the course of treatment of *E. coli* urinary tract infectious diseases by lowering polyamine synthesis in the patient by the use of aqueous pomegranate peel extract.

pH effect

Results showed that the use of citric acid buffer at different concentrations could inhibit the *E. coli* growth with no bactericidal properties. The current results indicate that the acidic property would not be a key factor for influencing the survival of *E. coli* and the aqueous pomegranate peel extract is emerging its antimicrobial properties due to its considerable polyphenol content as reported in several recent articles (38-40).

The aqueous pomegranate peel extract adjusted to pH=7 showed no bacteriostatic effect on *E. coli*. This could be explained by the effect of polyphenol oxidase (PPO). PPO is the main enzyme involved in the oxidation of phenolic compounds and its activity is pH dependent (41). This reaction is called enzymatic browning and occurs readily when the pH is between 5 and 7, while the activity of PPO is irreversibly inhibited at pH less than 3.5.

Conclusion

The aqueous pomegranate peel extract exhibited bacteriostatic, bactericidal and anti-virulence activities against urinary tract infectious *E. coli*. The use of the extract caused a reduction in the adhesion index accompanied with a reduction in motility and ornithine decarboxylation of the *E. coli* strains. The presence of phytochemicals including phenols, tannins and flavonoids may be responsible for these activities.

Further studies are required to identify and isolate the active compounds present in the pomegranate's peel which exhibits the antimicrobial effect and also to confirm these effects in vivo. The synergy between the extract active compounds and drug should be attentively studied which will probably solve the problem of multiple drug resistance, toxicity and overdose since when they combine a little concentration of two agents is required.

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