The effect of pomegranate peel extract and vitamin C in comparison with gemifloxacin on inhibiting adhesion of *Escherichia coli* to uroepithelial cells

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Zhian Ghazi Hussein * Nidhal AK Mohammed Ali **

Abstract

Background and objective: Prevention of bacterial adhesion is an attractive target for the development of new therapies in the prevention of bacterial infection. The aim of this study is to investigate the effects of pomegranate peel extract, vitamin C, combination of pomegranate peel extract and vitamin C & gemifloxacin on adhesion of *E.coli* to uroepithelial cells.

Methods: Uroepithelial cells were incubated with *E. coli* ATCC 25922 bacteria previously exposed to either the aqueous extract of pomegranate peel (AEPP), vitamin C, combination of both or gemifloxacin and the adherence was assessed by light microscopy.

Results: AEPP showed good antibacterial activity with minimum inhibitory concentration MIC of 25µg/ml and 10 mmol/ml for vitamin C and upon combination, the MIC was10µg/ml &5mmol/ml for AEPP & vitamin C respectively whereas for gemifloxacin was 0.03µg/ml. *In vitro* and *in vivo* adhesion of *E.coli* to uroepithelial cells were significantly inhibited by AEPP, vitamin C alone and in combination and by gemifloxacin.

Conclusion: AEPP showed good antibacterial activity, inhibited *E.coli* adhesion to uroepithelial cells and were potentiated by vitamin C.

Keywords: pomegranate, vitamin C, gemiflxacin, adhesion.

Introduction

Attention has been drawn to the antimicrobial activity of plants and their metabolites due to the challenge of growing incidences of drug-resistant pathogens[1]. Pomegranate has long history of medicinal uses owing its activity to the polyphenols constituents[2]. The antibacterial activity of pomegranate has been studied against various organisms[3,4]. Synergism was obtained upon combination of pomegranate with cupper, ferric ion and vitamin C[5] . Infections related to the insertion of medical devices in the body are common especially in relation to urinary catheterization, where the process involves the adhesion of urethral organisms onto the devices, multiplication of the organisms, biofilm formation, and the seeding of the bladder mucosa and urine by plank tonic bacteria[6]. The selection of different effective agents to inhibit bacterial adhesion must take into account their activities against the causative organisms otherwise the treatment will be not effective[7]. At least these agents must have activity against *E.coli* which is one of the most pathogenic organism involved in urinary tract infections[8]. Gemifloxacin is a fluoroquinolones antibiotic with potent broad spectrum activity against members of the family Enterobacteriaceae and gram-positive organisms[9,10]. It penetrates bacterial cell walls and inhibits DNA gyrase activity, rapidly kills susceptible organisms[11]. In subinhibitory concentrations, gemifloxacin found to inhibit bacterial virulence factors[12]. In view of these propositions we intended to compare the effects of pomegranate peel extract and vitamin C separately and in combination to that of gemifloxacin on both the *in vitro* and in vivo adhesion of *E.coli* to uroepithelial cells.

* Ministry of Health, Kurdistan Region, Erbil, Iraq
** Department of Pharmacology, College of Medicine, Hawler Medical University, Erbil, Iraq.

Methods

Extraction of the plant

Fresh pomegranate was collected from Iraqi Kurdistan region and was defined (*punica granatum L*.) in the department of plants, College of Agriculture, University of Salahadeen. Fifteen grams of fresh pomegranate peel was washed with distilled water & blended with 45ml of distilled water. The crude extract was filtered by Whatman filter No.1 and evaporated at reduced pressure in rotary evaporator at 45˚C.

Screening for antibacterial activity of pomegranate peel extract (AEPP)

The antibacterial activity of AEPP was tested by agar diffusion method according to the method of Ahmad and Beg[13] as follows: Bacterial suspension (10^6 CFU/ml) of *E.coli* ATCC 25922 was prepared in normal saline from an overnight culture, compared with 0.5 McFarland tubes and cultured onto Muller Hinton agar. AEPP, vitamin C and gemifloxacin were prepared in different concentrations & tested for their antibacterial activity. Gemifloxacin used as positive standard to determine the sensitivity of the tested microorganism. Each test was performed three times. The plates were then incubated at 37˚C for 18-24 hours and the mean diameter of zone of inhibition was calculated.

Determination of MIC:

The MIC of AEPP, vitamin C each alone and in combination with gemifloxacin was evaluated using the microdilution broth method according to National Committee for Clinical Laboratory Standards[14].

Adhesion assay

In vitro assay: The *in vitro* adherence of *E.coli* to uroepithelial cells was studied according to the method of Suzanne *et al* [15]. Fresh urine was collected from normal healthy women with no history of urinary or vaginal infections who are not taking contraceptive or antimicrobial agents. The urine was immediately centrifuged at 4000rpm for 15 minutes, the supernatant was discarded & the uroepithelial cells were harvested by washing the sediment three times with 5ml of phosphate buffer saline (pH 7.2). An epithelial cell count of $2X10^5$ cells /ml was obtained by re-suspending a suitable number of the epithelial cell in phosphate buffer saline pH 5 adjusted by light microscopy. One ml of bacterial suspension ($1X10^8$ CFU) 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, alcohol fixed and stained with toludine blue stain (0.1%) for 10 minutes and examined under light microscopy (X100).The mean number of *E.coli* adhering to the first 50 uroepithelial cells were counted and the standard error of mean was calculated for each preparation. All tests were performed three times using the MIC value for AEPP, vitamin C alone and in combination with gemifloxacin. *In vivo* adhesion assay: Female domestic rabbits (*oryctologus cuniculus*) weighing between 1-2 kg were used. The rabbits were kept in the animal house at 25˚C room temperature and fed vegetables and barley freely. The rabbits were anesthetized by injecting ketamine (35mg/kg) & xylazine (5mg/kg) intraperitoneally[16]. An incision was made in the lower midline aiming to reach urinary bladder. The bacterial suspension, ($1X10^8$ CFU /ml) either alone or treated with different concentration of either AEPP, vitamin C alone, in combination or gemifloxacin were introduced into the urinary bladder according to the method of Aronsonm *et al*[17]. Thereafter, the incision was sutured and the rabbits were kept under observation. Four rabbits were used for each treatment group; one rabbit was considered as control negative (only bacterial suspension) and the others were considered as treated by the MIC value of AEPP, vitamin C, each alone or in combination or gemifloxacin. Statistical analyses of the results were carried out using SPSS (ver.18) and the

data were analyzed by a one way ANOVA and Tukey's multiple comparison test.

Results

Screening for antibacterial activity of AEPP

The AEPP showed antibacterial activity against *E.coli* ATCC 25922 which was exhibited by the formation of 14 and 25 mm zone of inhibition when 62.5 and 250µg/ml of AEPP was tested respectively by the agar diffusion method.

Minimum inhibitory concentration of AEPP & vitamin C

The MIC of the AEPP, vitamin C obtained was 25 µg/ ml and 10 mmol respectively. whereas for the combination of AEPP and vitamin C was 10µg/ml and 5mmol respectively. Gemifloxacin produced 0.03µg/ml MIC.

Inhibition of *in vitro* and *in vivo* adhesion to uroepithelial cells

Figure 1 shows the effect of AEPP, vitamin C, combination of AEPP with vitamin C and gemifloxacin on *in vitro* adhesion of *E.coli* to uroepithelial cells. AEPP and vitamin C at 25 µg/ml and 10 mmol/ml significantly (P<0.001) inhibited the adhesion of *E.coli* ATCC 252922 to uroepithelial cells by reducing the number of attached bacteria per cell to12.3 ±1.179 and 11.88 ±1.656 when compared to control **(**43.34±5.48**)**. Combination of AEPP and vitamin C showed significant (P<0.001) antiadhesive effect when 5mmol/ml vitamin C was combined with 10 µg/ml which reduced the attached bacteria to the epithelial cells from 43.34 ±5.48 in control to 17.2 ±1.59 in combination treatment groups. Gemifloxacin at MIC significantly reduced (P<0.001) attached bacteria to 22.4 ±1.766 when compared to control group. The adhesion of *E.coli* to the urinary bladder epithelial cells of rabbits was reduced significantly (P<0.001) by AEPP, vitamin C, combination of both and gemifloxacin, Figure 2.

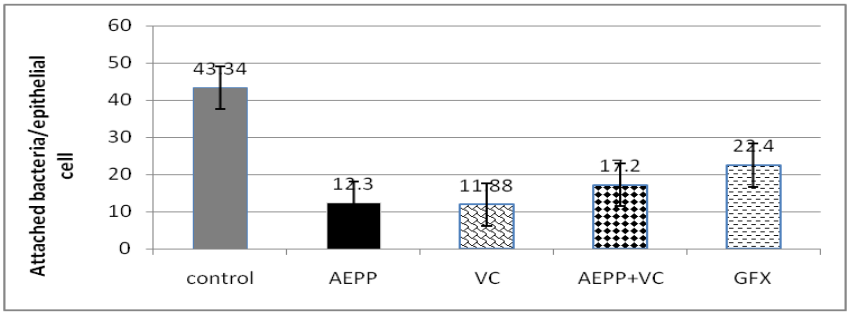

Figure 1: The effect of aqueous extract of pomegranate peel extract (AEPP), vitamin C (VC), combination of AEPP with vitamin C and gemifloxacin (GFX) on *in vitro* adhesion of *E.coli* to uroepithelial cells.

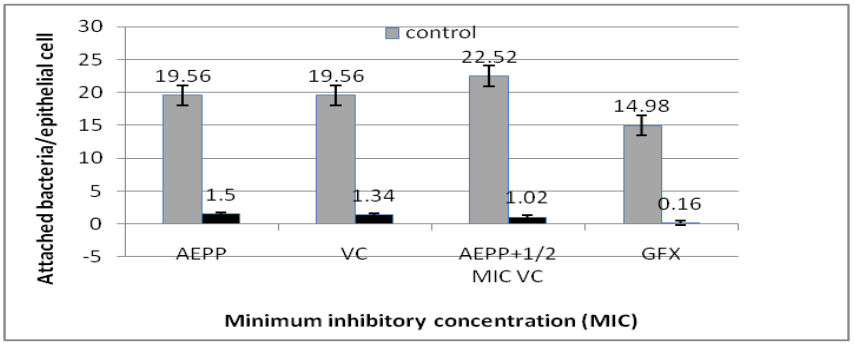

Figure 2: The effect of aqueous extract of pomegranate peel (AEPP), vitamin C (VC), combination of AEPP with vitamin C and gemifloxacin (GFX) on *in vivo* adhesion of *E.coli* to urinary bladder epithelial cells of rabbits.

Discussion

Pomegranate juice is growing in popularity in most countries[18,19]. The antibacterial activity of AEPP in high concentration shown in this study was comparable to that of gemifloxacin, which indicates its potent antibacterial activity against this bacterial strain. The determined MIC value of AEPP (25 µg/ ml) was close to those reported for *E.coli* by [20,21,22] although other studies reported lower or higher MIC values which could be related to differences in the method of extraction and the source of pomegranate fruit[23,24]. This antimicrobial activity of AEPP against *E. coli* could most probably be related to its oxidizing property [25] since the active compositions of pomegranate peel (phenolic acids) act as oxidizing antimicrobial agents [2,26,27] or that the antimicrobial activity could be related to the tendency of tannin rich ellagitannins constituents, to bind irreversibly to microbial DNA and cell membrane[28] thereby affecting bacterial growth & multiplication[29]. Such proposition has been claimed for tannins against different micro-organisms[30,31]. The lower MIC of vitamin C found in this study than those reported by Supayang *et al* [32] might be due to the differences in *E. coli* strains. The antibacterial effect of vitamin C is explained on the basis of its low molecular weight and hydrophilic nature that can readily cross the bacterial cell membrane through specific transporters on the bacterial cell membrane which enables it to reveal the antioxidant effect as antimicrobial compound[33]. The potentiating antibacterial activity of AEPP after combination with vitamin C shown by the reduction in the MIC values by more than 2 folds, suggest that vitamin C is enhancing the antimicrobial effect of pomegranate. This potentiating effect was reported by[5,34] whom explained the synergistic effect between pomegranate and vitamin C to enhance the stabilizing property of vitamin C. It is worth noting that Pomegranate peel is a rich source of vitamin C[19,21,35]. The MIC of gemifloxacin obtained in this study is comparable to those reported by Ansgar and Peter[36] although being lower, which most probably related to differences in the bacterial strain The significant *in vitro* and *in vivo* antiadhesive property of AEPP found in this study deciphers its interference with bacterial virulence factors (fimbrai and pilli) that aids the attachment of bacteria to epithelial cells. This property could be related to ellagitannins tannins constituents that have the ability to interact with the complex bacteria structures and prevent their adhesion[37]. The antioxidant property of vitamin C could explain its potent antiadhesive effect found in this study and illustrates the importance of vitamin C in preventing bacterial adhesion and urinary tract infections[38]. This explains the synergism upon combination with AEPP whereby both, *in vitro* and *in vivo* adhesion of *E.coli* to the uroepithelial cells was inhibited to a greater extend. Gemifloxacin inhibition of *E.coli* adhesion seen in the present study has also been reported by Mandell[10] which demonstrate its ability in reducing bacterial fimbriation parameters. In conclusion, The inhibitory property of pomegranate peel against *E. coli* adhesion worth to be taken into consideration. This effect is similar to cranberry juice which is recommended in the prophylactic use of urinary tract infections[39].

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