Journal of Pharmacy & Pharmacognosy Research
E-ISSN: 0719-4250
editor@jppres.com
Asociación de Académicos de Ciencias Farmacéuticas de Antofagasta
Chile

Oluwole Osungunna, Michael; Onawunmi, Grace O.
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Journal of Pharmacy & Pharmacognosy Research, vol. 4, núm. 1, enero-febrero, 2016, pp. 25-38
Asociación de Académicos de Ciencias Farmacéuticas de Antofagasta
Antofagasta, Chile

© 2016 Journal of Pharmacy & Pharmacognosy Research, 4 (1), 25-38
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Anti-adhesion and antibiotic modulatory evaluation of grapefruit (*Citrus paradisi*) juice and seed extract on bacteria isolated from urine and catheter

[Anti-adhesión y evaluación moduladora de antibióticos de jugo y extracto de semilla de pomelo (*Citrus paradisi*) sobre bacterias aisladas de orina y catéter]

Michael Oluwole Osungunna[1*] and Grace O. Onawunmi[2]

Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.
*E-mail: mowole@oauife.edu.ng, yomosun2002@yahoo.co.uk

Abstract

Context: The *in vivo* use of grapefruit seed in the treatment of urinary tract infections (UTIs) has been reported but the mechanism of action is yet to be explained.

Aims: Evaluate the anti-adhesion and antibiotic modulatory activities of grapefruit seed extract and juice as their possible mechanisms of action.

Methods: Sub-inhibitory concentrations of 2.5 and 5 mg/mL as well as 10.3 and 5.15 mg/mL of grapefruit seed extract and juice respectively were evaluated for modulatory activity of ciprofloxacin, streptomycin and nalidixic acid against one hundred and twenty seven bacterial isolates from mid-stream urine (MSU) (100), catheter-stream urine (CSU) (14) and catheter tips (CT) (13) using the agar dilution method. Anti-adhesion activity of grapefruit seed extract and juice at sub-inhibitory concentrations of 2.5 and 1.03 mg/mL respectively was evaluated against twenty three (23) moderately adherent bacterial isolates from MSU (10), CSU (7) and CT (6) using the tissue culture plate method.

Results: The results revealed that grapefruit juice (5.15 mg/mL) showed more effect on nalidixic acid activity than seed extract (2.5 mg/mL). Grapefruit juice showed more anti-adhesion activity than grapefruit seed extract at the concentration tested.

Conclusions: The study concluded that grapefruit seed extract and juice had anti-adhesion and antibiotic modulatory effects on bacteria associated with UTIs.

Keywords: Anti-adhesion; catheter-stream urine; catheter tips; mid-stream urine; modulatory effect; urinary tract infections.

Resumen

Contexto: El uso *in vivo* de la semilla de pomelo se ha informado en el tratamiento de la infecciones del tracto urinario (ITU), pero aún no se ha explicado el mecanismo de acción por el que ocurre este beneficio.

Objetivos: Evaluar la anti-adherencia y actividades moduladoras de antibióticos del extracto de semilla y el zumo de pomelo como sus posibles mecanismos de acción.

Métodos: Concentraciones sub-inhibitorias de 2,5 y 5 mg/mL, así como 10,3 y 5,15 mg/mL de extracto de semilla de pomelo y el jugo, respectivamente fueron evaluados para demostrar la actividad moduladora de la ciprofloxacina, estreptomicina y ácido nalidíxico en contra de ciento veintisiete aislamientos bacterianos desde orina corriente (MSU) (100), catéter de orina (CSU) (14) y puntas de catéter (CT) (13) utilizando el método de dilución en agar. La actividad anti-adherencia del extracto de semilla y el jugo de pomelo a concentraciones sub-inhibitoria de 2,5 y 1,03 mg/mL, respectivamente, se evaluó frente a veintitrés (23) aislamientos bacterianos moderadamente adherentes a partir de MSU (10), CSU (7) y CT (6) utilizando el método de la placa de cultivo de tejidos.

Resultados: Los resultados revelaron que el zumo de pomelo (5,15 mg/mL) mostró más efecto sobre la actividad del ácido nalidíxico que el extracto de semilla (2,5 mg/mL). El jugo mostró una mayor actividad anti-adhesión que el extracto de semilla de pomelo a la concentración probada.

Conclusiones: El extracto de semilla y el jugo de pomelo tuvieron efectos anti-adherente y moduladores de antibióticos en las bacterias asociadas con las infecciones urinarias.

Palabras Clave: Anti-adhesión; catéter de flujo de orina; efecto modulador; flujo medio de orina; infecciones del tracto urinario; punta de catéter.

ARTICLE INFO
Received | Recibido: November 10, 2015.
Received in revised form | Recibido en forma corregida: December 24, 2015.
Accepted | Aceptado: December 26, 2015.
Available Online | Publicado en Línea: January 18, 2016.
Declaration of interests | Declaración de Intereses: The authors declare no conflict of interest.
Funding | Financiación: The authors confirm that the project has not funding or grants.
Academic Editor | Editor Académico: Gabino Garrido.

INTRODUCTION

Controversies have continued to trail the presence or otherwise of antimicrobial activity in grapefruit seed extract. While some researchers have reported the presence of antimicrobial activity *in vitro*, some have reported otherwise (Reagor et al., 2002; Adedeji et al., 2007). However, it was observed that those that reported activity made use of commercial grapefruit seed extract to which their reported activity had been linked to the presence of benzethonium chloride (von Woedtke et al., 1999) while those that reported no activity made use of crude extract of grapefruit seed. Authors that reported activity with the crude extract of grapefruit seed however made use of 70% ethanol as vehicle for reconstitution of the extract (Cvetnic and Vladimir-Knezevic, 2004). Al-Ani et al. (2011) also reported activity with the aqueous extract of grapefruit seed extract with no activity in the ethanolic extract.

Some clinical observations on the use of grapefruit seeds in the treatment of urinary tract infections, which suggested an *in vivo* antibacterial characteristic of dried or fresh grapefruit seeds when taken at a dosage of five to six seeds every eight hours for up to two weeks that was comparable to that of proven antibacterial drugs have been reported (Oyelami et al., 2005). The reversal of antibiotic resistance pattern after two weeks' treatment with grapefruit seeds was also reported. The in vivo report suggested that grapefruit seeds had (i) antimicrobial activity (ii) antibiotic modulatory activity or (iii) that there are other ways by which grapefruit seeds may exhibit the reported clinical observations other than that obtained by the conventional agar diffusion method.

This study therefore aimed at evaluating grapefruit seed extract and juice for their anti-adhesion and antibiotic modulatory activities as possible mechanisms of observed clinical action since it has been established that adhesion of organisms to uroepithelial cells is an important step in the pathogenesis of infections as urinary tract infections and agent(s) that can prevent this adhesion may be of clinical importance in the management of such infections.

MATERIAL AND METHODS

Plants collection and identification

Leaves and fruits of grapefruits (*Citrus paradisi*) were collected from Jagba farm in Gbongan, Ayedaade Local Government Area of Osun State. The plant was identified and authenticated at the Forestry Research Institute of Nigeria, Ibadan, Oyo State, Nigeria. The authenticated plant has the Voucher number FHI 108842.

Preparation of grapefruit seed extract and juice

Grapefruits were collected from an identified source between February and May, 2012 and processed with a view to collecting the juice, and seeds. The fruits were cut into two halves using a sharp knife. The juice was expressed into a sterile container, lyophilized, using a freeze-drier and stored in amber bottle placed in desiccator to preserve until needed. The percentage yield was calculated.

The seeds of the grapefruits were air-dried at room temperature (25°C) for at least two weeks after which they were ground into powder form using a grinder (Daiki, Japan). The powdered seeds (383 g) was extracted by macerating in 1.5 liter 70% ethanol for 72 hours under regular shaking, using a rotary flask shaker (Griffin, U.S.A). The extract was filtered using Whatman No 1 filter paper and filtrates concentrated in rotary evaporator (Buchi, Switzerland). The percentage yield was calculated.

Collection of mid-stream urine isolates

After ethical approval for the study was obtained from the OAUTHC Research and Ethical Committee, consecutive clinical isolates from patients presenting with Urinary Tract Infections (UTIs) were collected from Microbiology Laboratory of Obafemi Awolowo University Teaching Hospital Complex on agar slopes in McCartney bottles between August and December, 2012 and brought to the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutics, Obafemi Awolowo University, Ile - Ife for identification and

authentication using conventional biochemical tests.

Collection of catheter specimen

Prior to catheter change or removal from each patient, 10 mL of urine was obtained from the distal edge of the catheter tube (after cleaning with Savlon antiseptic by Johnson and Johnson, South Africa) using a sterile needle and syringe into sterile universal container (Kunin and McCormack, 1966; Kunin, 1979) from Urology Unit of Obafemi Awolowo University Teaching Hospital Complex and transported to the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutics, Obafemi Awolowo University, Ile-Ife, for analysis. The tip of the catheter removed from each patient was cut with a sterile surgical blade into sterile universal container and similarly brought to the same laboratory for analysis. This was done between July and August, 2013 during which a total of sixty-four (64) catheter tips were collected.

Isolation from catheter-stream urine (CSU) and catheter tips

After receipt of the urine in the laboratory, a sterile calibrated wire loop was used to deliver a loopful (0.01 mL) of urine onto Cysteine Lactose-Electrolyte-Deficient (CLED) (Lab M, Bury, UK) agar plates, streaked and incubated aerobically at 37°C for 24 hours. The isolated colonies were characterized using conventional biochemical tests.

Ten mL sterile nutrient broth (Oxoid) was added to the catheter tip in sterile universal container with aseptic precautions and incubated at 37 °C for 24 hours. The resulting culture was then vortexed for about five minutes after which a loopful was streaked on CLED agar plates and incubated aerobically at 37°C for 24 hours. The isolated colonies were characterized using conventional biochemical tests such as Gram's stain, Indole, citrate, catalase, Methyl red -Voges-Proskauer (MRVP) test, urease, oxidase and hydrogen sulphide production, among others (Barrow and Feltham, 2003).

Determination of minimum inhibitory concentrations of streptomycin, nalidixic acid and ciprofloxacin against clinical bacterial isolates

The lowest concentration that prevented the growth of an organism either in nutrient broth or in agar after a specified period of incubation was taken as the Minimum Inhibitory Concentrations (MIC) of that antimicrobial agent. MIC of streptomycin was determined by the agar dilution method as approved and described (EUCAST, 2000). Equivalent of 1000 mg of streptomycin (Sigma-Aldrich, USA) was dissolved in 1000 mL of sterile distilled water to give 1 mg/mL concentration. From this was prepared other concentrations of 0.03125, 0.0625, 0.125, 0.25, and 0.5 mg/mL of streptomycin. A 1:10 dilution of each concentration was made by adding 1 mL each of the concentration to 9 mL of Mueller-Hinton agar (Oxoid, UK). This was done in duplicate and poured into sterile Petri dishes (Axiom, China).

A distinct colony of each isolate to be tested was incorporated in 2 mL of sterile nutrient broth (Oxoid, England). Tubes were incubated at 37°C overnight. A 1:1000 dilution of the nutrient broth suspension was performed in sterile nutrient broth to give approximately 1.0 x 10^5 CFU/mL of microorganisms for MIC determination. The diluted suspensions (100 µL) were subsequently transferred into the wells of a sterile microtitre plate with the use of a micro pipette. Inoculation of plates was carried out by dipping the points of the already sterilized multi-inoculator into the wells containing the diluted isolates and placing gently but firmly on the surface of oven-dried agar plates containing different concentrations of streptomycin with *E. coli* ATCC 25922 as control. Results were taken after 24 hour incubation at 37°C. The concentration at which the inoculation points showed no growth was taken as the MIC. The procedures were repeated for nalidixic acid (Biomedical, Germany) and ciprofloxacin (Juhel Pharmaceuticals Nigeria, Ltd) and the experiments done in duplicates.

Determination of minimum inhibitory concentration of grapefruit seed extract and grapefruit juice using the agar dilution method

An equivalent of 1 g/mL concentration of grapefruit seed extract was prepared by accurately weighing 10 g grapefruit seed extract and dissolved in 10 mL sterile distilled water in McCartney bottle from where concentrations of 125, 250, and 500 mg/mL concentrations were prepared. 1:10 dilution of each concentration was made by adding 1 mL to 9 mL sterile nutrient agar (Oxoid, UK). This gave final concentrations of 12.5, 25, 50, and 100 mg/mL in agar. This was done in duplicate and poured into sterile Petri dishes (Axiom, China).

A distinct colony of each isolate to be tested was incorporated in 2 mL of sterile nutrient broth (Oxoid, England). Tubes were incubated at 37°C overnight. A 1:1000 dilution of the nutrient broth suspension was performed in sterile nutrient broth to give approximately 1.0 x 10^5 CFU/mL of microorganisms for MIC determination. The diluted suspensions (100 µL) were subsequently transferred into the wells of a sterile microtitre plate with the use of a micro pipette. Inoculation of plates was carried out by dipping the points of the already sterilized multi-inoculator into the wells containing the diluted isolates and placing gently but firmly on the surface of oven dried agar plates containing different concentrations of grapefruit seed extract with *E. coli* ATCC 25922 as control. Results were taken after 24 hour incubation at 37°C. The concentration at which the inoculation points showed no growth was taken as the MIC.

The procedures were repeated for grapefruit juice with final concentrations of 12.875, 25.75, 51.5, and 103 mg/mL respectively and the experiments done in duplicates.

Determination of minimum inhibitory concentrations by microtitre broth dilution method

This was done as described (Amsterdam, 1996). Briefly, 100 µL of Mueller-Hinton (Oxoid) broth was dispensed into all wells of a 96-wells microtitre plate using the micropipettor. Grapefruit seed extract (100 µL each of 100 mg/mL) and 103 mg/mL grapefruit juice was pipetted into the first well (column 1) of rows A and B respectively followed by sucking up and down six times by means of micropipettor to mix. To column 1 of rows C, D and E was added 100 µL of 1 mg/mL of ciprofloxacin, streptomycin and nalidixic acid respectively as positive controls. The mixture (100 µL) was withdrawn from column 1 and added to column 2 to make a twofold dilution of column 1. The resulting mixture in column 2 was mixed up and down six times before 100 µL was transferred into column 3. The procedure was repeated down to column 10. The mixture (100 µL) withdrawn from column 10 was however discarded rather than putting it in column 11.

Overnight grown culture (100 µL) of test organism was transferred into 9.9 mL sterile water in McCartney bottle to make 1:100 dilution from where 100 µL was transferred into another 9.9 mL sterile water in McCartney bottle. The final dilution gave about 2 x 10^4 CFU/mL. Five µL of the final dilution of the test organism was added to each well except column 12, which served as negative control. The plate was incubated at 37°C for 24 hours. Each well of the plate was examined for the presence or absence of growth by addition of 20 µL MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide solution to each well.

Determination of antibiotic modulatory activity of grapefruit seed extract and grapefruit juice

About 1 mL of each concentration of 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL of test antibiotic and 1 mL of 50 mg/mL grapefruit seed extract was added to 8 mL of sterile melted nutrient agar (Oxoid, UK) to give 1:10 dilution of each concentration. This was done in duplicate and poured in sterile Petri dishes (Axiom, China).

A distinct colony of each isolate to be tested was incorporated in 2 mL of sterile nutrient broth (Oxoid, England). Tubes were incubated at 37°C overnight. A 1:1000 dilution of the nutrient broth suspension was performed in sterile nutrient broth to give approximately 1.0 x 10^5 CFU/mL of microorganisms for MIC determination. One hundred µL of the diluted suspensions were subsequently transferred into the wells of a sterile microtitre plate with the use of a micro pipette. Inoculation of

plates was carried out by dipping the points of the already sterilized multi-inoculator into the wells containing the diluted isolates and placing gently but firmly on the surface of oven dried agar plates containing different concentrations of grapefruit seed extract with *E. coli* ATCC 25922 as control. Results were taken after 24 hour incubation at 37°C. The concentration at which the inoculation points showed no growth was taken as the MIC. The procedures were repeated using 1 mL of 25 mg/mL grapefruit seed extract and 1 mL of each concentration of 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL of test antibiotic.

Two controls were used for these tests. The first control aimed at testing the viability of the organisms as the plate contained neither the antibiotic nor the extract while the second control aimed at confirming that the concentration of the extract used has no inhibitory effect on the test organisms as the plate was prepared by adding 1 mL of the tested concentration of the extract to 9 mL nutrient agar (Oxoid, UK). The effect of the added grapefruit seed extract on the MIC of the antibiotic tested was noted.

The procedures were repeated using 51.5 mg/mL and 103 mg/mL stock solutions of grapefruit juice respectively and the experiments done in duplicates.

Determination of adherence property of the isolates

This was determined using the tissue culture plate method as described (Christensen et al., 1982). Briefly, 10 mL of Trypticase soy broth (Oxoid, UK) with 1% glucose (Kermel, China) was inoculated with a loopful of test organism from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 hours. The culture was further diluted 1:100 with fresh medium. Flat bottom tissue culture plates of 96 wells (Greiner, Germany) were filled with 200 µL of diluted cultures individually. Only sterile broth was used as blank and negative control. The control organism was equally diluted and incubated. The culture plates were incubated at 37°C for 24 hours. After incubation, the plates were tapped and wells washed with 200 µL of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms, which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet (Kemlight, China). Excess stain was washed with deionized water and plates dried properly. Optical densities (OD) of stained adherent biofilm were obtained with microplate reader (DNM-9602, Beijing Perlong New Technology Co., Ltd, China) at wavelength 570 nm. Experiment was repeated thrice and average OD values of sterile medium calculated and subtracted from all test values. The results were interpreted based on OD values as Non/weak if OD < 0.120, moderate if OD is between 0.120 and 0.240, and strong if OD > 0.240.

Determination of anti-adhesion activity of grapefruit seed extract and grapefruit juice

To each well of A1 – A12 of a flat bottom microtitre plate (Greiner, Germany) was added 200 µL of sterile Trypticase Soy Broth (Oxoid, UK) supplemented with 1% glucose as blank and negative control. Sterile trypticase soy broth supplemented with 1% glucose (180 µL) was put in each well of B1 – B12 and to three consecutive wells in the row was added 20 µL of the same test organism. Sterile trypticase soy broth supplemented with 1% glucose (160 µL) was added to each well of C1 – C12 and to three consecutive wells in the row was added 20 µL each of the test organism and 25 mg/mL seed extract (pH 3.8). However, D1 – D12 was treated as C1-C12 only that the seed extract used in D row has been treated with 1 M NaOH with a view to bringing its pH to a neutral value of 7.1. The microtitre plate was then incubated at 37°C for 24 hours, washed thrice with phosphate buffer saline (pH 7.2), fixed with 2% sodium acetate and stained with 0.1% crystal violet. The OD was read in microplate reader (DNM-9602, Beijing Perlong New Technology Co., Ltd, China) and the effect of seed extract on adherent property of the organism evaluated.

The procedures were repeated using 10.3 mg/mL stock solution of grapefruit juice and the experiments done in triplicates.

Statistical analysis

The effects of grapefruit seed extract and juice on adhesion property were analyzed with one way analysis of variance (ANOVA) followed by Tukey's

pairwise multiple comparison test on GraphPad Prism. The effect was considered significant at $p < 0.05$.

RESULTS

The MICs of the grapefruit seed extract and juice against moderately adherent isolates selected for anti-adhesion study as well as their identities were shown in Table 1. These isolates were selected based on their adhesion property.

The MICs of grapefruit seed extract and juice as well as some selected antibiotics against some typed strains bacteria as determined by broth microdilution method were as shown in Table 2.

The results of the antibiotic modulatory activity of sub-inhibitory concentrations of grapefruit seed extract and grapefruit juice on ciprofloxacin, streptomycin and nalidixic acid can be classified into (i) antibiotics that have their MICs decreased, an indication of increased antibiotic activity (ii) those that have their MICs increased, an indication of decreased antibiotic activity and (iii) those whose MICs were not affected, an indication of no effect on antibiotic activity.

The effect of grapefruit seed extract on antibiotic activity varied with the antibiotic, concentration of the grapefruit seed extract and the source of the isolate. The mid-stream urine isolates (25 – 27%) showed reduced MIC with all the antibiotics tested in the presence of 2.5 mg/mL of grapefruit seed extract. Increasing the concentration to 5 mg/mL reduced the effect of grapefruit seed extract against ciprofloxacin and streptomycin but increased the effect on nalidixic acid as shown in Table 3.

Grapefruit seed extract (2.5 mg/mL) showed no effect on the activity of the three antibiotics against catheter-stream urine isolates, while the MIC of only nalidixic acid was reduced at 5 mg/mL concentration.

Grapefruit seed extract at 2.5 mg/mL reduced the MIC of ciprofloxacin and nalidixic acid against some catheter tip isolates (15.38%) and had no effect on streptomycin. Grapefruit seed extract at 5 mg/mL increased the activity of only nalidixic acid and had no effect on the MIC of the others.

Grapefruit juice at 1:10 dilution (10.3 mg/mL) decreased the MIC of all the antibiotics against all the bacterial isolates tested as follows: mid-stream urine isolates (40 – 87%), catheter- stream urine isolates (28.57 - 78.57%) and catheter tip isolates (61.5 – 100%). Decreasing the concentration of the grapefruit juice to 5.15 mg/mL (1:20 dilution) resulted in a reduction in the observed effect of grapefruit juice on the MIC of the antibiotics as shown in Table 4.

The anti-adhesion activity of grapefruit seed extract and juice were assessed against selected moderately adherent bacterial isolates from the mid – stream urine, catheter stream urine and catheter tips. At a concentration of 2.5 mg/mL grapefruit seed extract, 25% of the isolates tested showed reduced adhesion. However, when the acidic pH of the extract was neutralized, the anti-adhesion effect was reduced to 16.7% as shown in Figs. 1 and 2. Grapefruit juice at a concentration of 1.03 mg/mL (1:100 dilution) reduced adhesion in 62.5% of the isolates while neutralized grapefruit juice reduced adhesion in 37.5% of the tested isolates as shown in Figs. 3 and 4.

DISCUSSION

A number of *in vitro* studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the MICs of the antibiotics against some resistant strains (Darwish et al., 2002; Al-Hebshi et al., 2006; Betoni et al., 2006). These studies have provided the basis for understanding the action of plant antimicrobials. There is reason therefore to believe that, plants could be a source of compounds that can increase the sensitivity of bacterial cells to antibiotics. Such compounds could be useful particularly against antibiotic resistant strains of pathogenic bacteria. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored. The curative effect of plant extracts in combination studies have been variably referred to as resistance modifying/modulating activity (Gibbons et al., 2004). This ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics (Zhao et al., 2001; Lewis and Ausubel, 2006).

The antibiotic modulatory effect of grapefruit (*Citrus paradisi*) seed extract and juice on three selected antibiotics was investigated in this study. The effects produced include (i) decrease in MICs (ii) increase in MICs and (iii) no change in MICs.

Decreased MICs indicated that there was an increase in the activity of the antibiotics when combined with either grapefruit seed extract or grapefruit juice. The increased activity detected in this study was not specific to any group of organisms or class of antibiotics. This suggests that grapefruit seed extract and juice contain a mixture of compounds that can enhance the activity of different antibiotics. Grapefruit has been reported to contain a number of polyphenolic compounds, including the flavanone, naringin, alongside the two furanocoumarins, bergamottin and dihydrobergamottin (He et al., 1998).

Table 1. Minimum Inhibitory Concentrations (MICs) of grapefruit juice and seed extract against selected bacterial isolates used for anti-adhesion activity evaluation.

CODE	MIC of grapefruit juice (mg/mL)	MIC of grapefruit seed extract (mg/mL)	Organism identified	Adherence status as determined by tissue culture plate method
16819	12.88	100	*B. flexus*	Moderately adherent
16875	12.88	100	*K. pneumoniae*	Moderately adherent
16876	12.88	100	*B. flexus*	Moderately adherent
17079	12.88	100	*K. pneumoniae*	Moderately adherent
17085	12.88	50	*K. pneumoniae*	Moderately adherent
11808	103.00	100	*K. pneumoniae*	Moderately adherent
11224	25.75	100	*Pseudomonas cepacia*	Moderately adherent
13766	25.75	50	*Yersinia intermedia*	Moderately adherent
14007	25.75	50	*Enterobacter gergoviae*	Moderately adherent
14121	51.50	25	*K. pneumoniae*	Moderately adherent
11610	25.75	100	*Pseudomonas cepacia*	Moderately adherent
3^{CT}	ND	ND	*K. pneumoniae*	Moderately adherent
B_1^{CT}	ND	ND	*Aeromonas hydrophila*	Moderately adherent
B_5^{CT}	ND	ND	*K. pneumoniae*	Moderately adherent
B_{10}^{CT}	ND	ND	*Pseudomonas cepacia*	Moderately adherent
E_8^{CT}	ND	ND	*Yersinia intermedia*	Moderately adherent
E_{14}^{CT}	ND	ND	*Aeromonas hydrophila*	Moderately adherent
B_4^{CSU}	ND	ND	*Proteus vulgaris*	Moderately adherent
B_5^{CSU}	ND	ND	*K. pneumoniae*	Moderately adherent
B_{10}^{CSU}	ND	ND	*Aeromonas hydrophila*	Moderately adherent
B_{11}^{CSU}	ND	ND	*Proteus vulgaris*	Moderately adherent
B_{12}^{CSU}	ND	ND	*K. pneumoniae*	Moderately adherent

*ND = Not Determined

Table 2. Minimum Inhibitory Concentrations (MICs) of grapefruit seed extract, juice and selected antibiotics by broth microdilution method.

Samples	Minimum Inhibitory Concentrations (MICs) in mg/mL against				
	Conc. (mg/mL)	*Escherichia coli (ATCC 25922)*	*Staphylococcus aureus (NCTC 6571)*	*Pseudomonas aeruginosa (ATCC 10145)*	*Bacillus subtilis (NCTC 8263)*
Grapefruit seed extract	100	50	25	50	25
Grapefruit juice	103	25.75	25.75	12.875	25.75
Ciprofloxacin	1	0.00391	< 0.000977	< 0.000977	< 0.000977
Streptomycin	1	0.00781	< 0.001953	0.5	0.015625
Nalidixic acid	1	0.0625	0.0078125	0.125	0.25

Table 3. Antibiotic modulatory activity of grapefruit seed extract (GSE) on ciprofloxacin, streptomycin and nalidixic acid against bacterial isolates from the mid-stream urine (MSU), catheter-stream urine (CSU) and catheter tips (CT).

COMBINATIONS Antibiotics + GSE (mg/mL)	Source of the Isolates	EFFECT ON MICs		
		Decrease in MICs (%)	No change in MICs (%)	Increase in MICs (%)
Ciprofloxacin + GSE (2.5)	MSU (n = 100)	27 (27)	66 (66)	7 (7)
Ciprofloxacin + GSE (5)	MSU (n = 100)	7 (7)	39 (39)	54 (54)
Streptomycin + GSE (2.5)	MSU (n = 100)	25 (25)	55 (55)	20 (20)
Streptomycin + GSE (5)	MSU (n = 100)	3 (3)	48 (48)	49 (49)
Nalidixic acid + GSE (2.5)	MSU (n = 100)	27 (27)	69 (69)	4 (4)
Nalidixic acid + GSE (5)	MSU (n = 100)	38 (38)	55 (55)	7 (7)
Ciprofloxacin + GSE (2.5)	CSU (n = 14)	-	100 (14)	-
Ciprofloxacin + GSE (5)	CSU (n = 14)	-	100 (14)	-
Streptomycin + GSE (2.5)	CSU (n = 14)	-	64.3 (9)	35.7 (5)
Streptomycin + GSE (5)	CSU (n = 14)	-	64.3 (9)	35.7 (5)
Nalidixic acid + GSE (2.5)	CSU (n = 14)	-	100 (14)	-
Nalidixic acid + GSE (5)	CSU (n = 14)	42.9 (6)	57.1 (8)	-
Ciprofloxacin + GSE (2.5)	CT (n = 13)	15.4 (2)	46.1 (6)	38.5 (5)
Ciprofloxacin + GSE (5)	CT (n = 13)	-	76.9 (10)	23.1 (3)
Streptomycin + GSE (2.5)	CT (n = 13)	-	84.6 (11)	15.4 (2)
Streptomycin + GSE (5)	CT (n = 13)	-	84.6 (11)	15.4 (2)
Nalidixic acid + GSE (2.5)	CT (n = 13)	15.4 (2)	76.9 (10)	7.7 (1)
Nalidixic acid + GSE (5)	CT (n = 13)	61.5 (8)	38.5 (5)	-

Table 4. Antibiotic modulatory activity of grapefruit juice (GJ) on ciprofloxacin, streptomycin and nalidixic acid against bacterial isolates from the mid-stream urine (MSU), catheter-stream urine (CSU) and catheter tips (CT).

COMBINATIONS Antibiotics + GJ (mg/mL)	Source of the Isolates	EFFECT ON MICs		
		Decrease in MICs (%)	No change in MICs (%)	Increase in MICs (%)
Ciprofloxacin + GJ (5.15)	MSU (n = 100)	9.0 (9)	41.0 (41)	50.0 (50)
Ciprofloxacin + GJ (10.3)	MSU (n = 100)	40.0 (40)	46.0 (46)	14.0 (14)
Streptomycin + GJ (5.15)	MSU (n = 100)	18 (18)	43.0 (43)	39.0 (39)
Streptomycin + GJ (10.3)	MSU (n = 100)	81.0 (81)	8.0 (8)	11.0 (11)
Nalidixic acid + GJ (5.15)	MSU (n = 100)	48.0 (48)	52.0 (52)	-
Nalidixic acid + GJ (10.3)	MSU (n = 100)	87.0 (87)	13.0 (13)	-
Ciprofloxacin + GJ (5.15)	CSU (n = 14)	7.1 (1)	35.7 (5)	57.1 (8)
Ciprofloxacin + GJ (10.3)	CSU (n = 14)	28.6 (4)	50.0 (7)	21.4 (3)
Streptomycin + GJ (5.15)	CSU (n = 14)	7.1 (1)	71.4 (10)	21.4 (3)
Streptomycin + GJ (10.3)	CSU (n = 14)	42.9 (6)	42.9 (6)	14.3 (2)
Nalidixic acid + GJ (5.15)	CSU (n = 14)	35.7 (5)	64.3 (9)	-
Nalidixic acid + GJ (10.3)	CSU (n = 14)	78.6 (11)	21.4 (3)	-
Ciprofloxacin + GJ (5.15)	CT (n = 13)	7.7 (1)	15.4 (2)	76.9 (10)
Ciprofloxacin + GJ (10.3)	CT (n = 13)	69.2 (9)	30.8 (4)	-
Streptomycin + GJ (5.15)	CT (n = 13)	23.1 (3)	76.9 (10)	-
Streptomycin + GJ (10.3)	CT (n = 13)	61.5 (8)	38.5 (5)	-
Nalidixic acid + GJ (5.15)	CT (n = 13)	61.5 (8)	38.5 (5)	-
Nalidixic acid + GJ (10.3)	CT (n = 13)	100 (13)	-	-

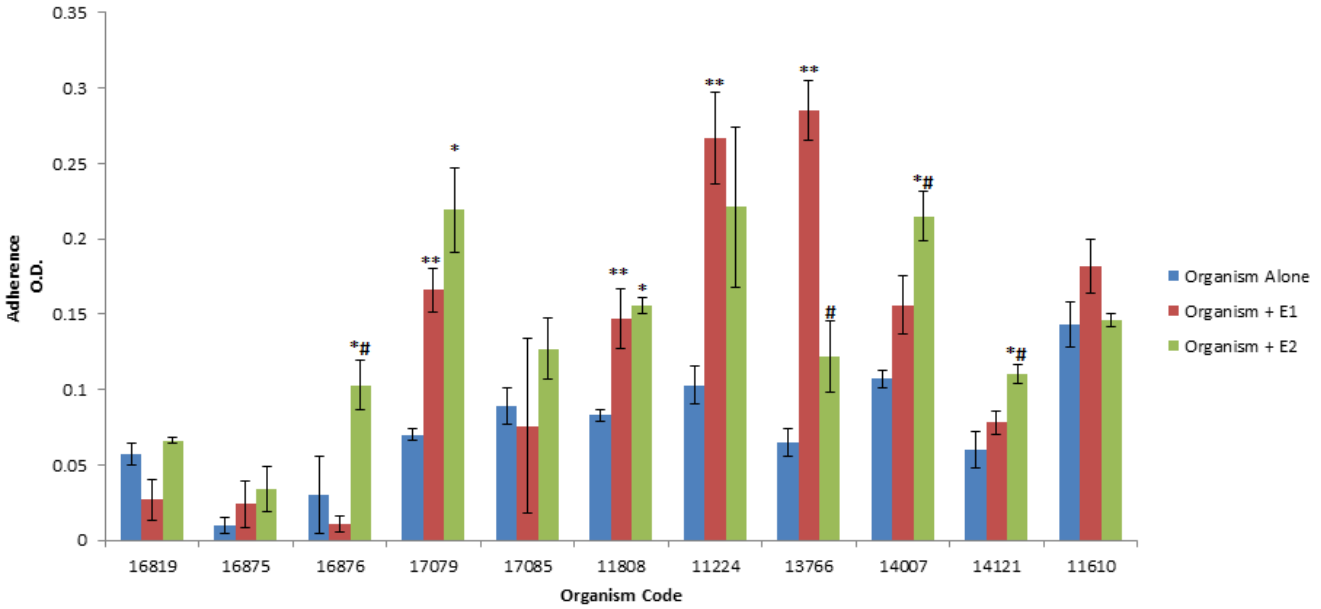

Figure 1. Anti-adhesion effect of 2.5 mg/mL grapefruit seed extract on bacterial isolates from Mid-stream urine.
E1 = crude grapefruit seed extract with pH 3.8; E2 = neutralized seed extract with pH 7.1. The bars represent mean of three data points and SD (Mean ± SD). P < 0.05 represents statistical differences between treated and untreated groups (one-way ANOVA followed by Tukey's pairwise multiple comparison). *p < 0.05 when the effect of neutral grapefruit seed extract was compared to the organism. #p < 0.05 when the effects of neutral grapefruit seed and crude grapefruit seed extract were compared. **p < 0.005 when the effect of crude grapefruit extract was compared to the organism.

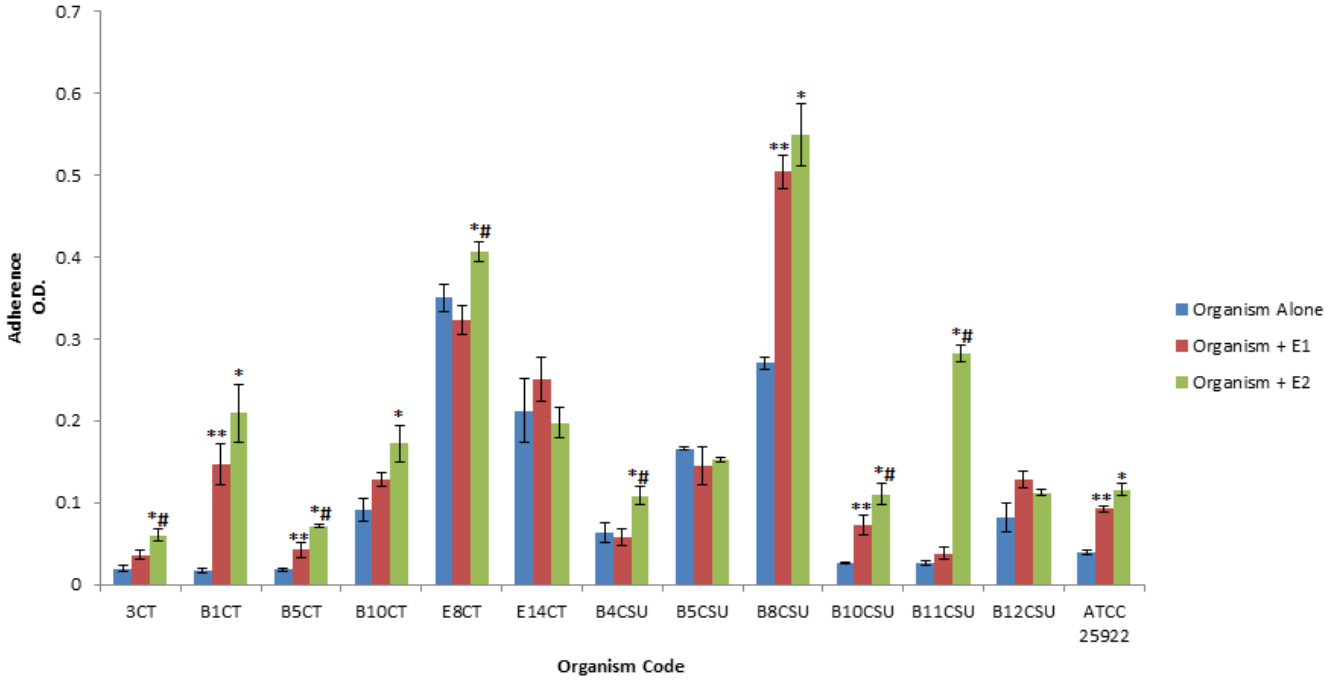

Figure 2. Anti-adhesion effect of 2.5 mg/mL grapefruit seed extract on bacterial isolates from catheter-stream urine (CSU) and catheter tips (CT).
E1 = crude grapefruit seed extract with pH 3.8; E2 = neutralized seed extract with pH 7.1. The bars represent mean of three data points and SD (Mean ± SD). P < 0.05 represents statistical differences between treated and untreated groups (one-way ANOVA followed by Tukey's pairwise multiple comparison). *p < 0.05 when the effect of neutral grapefruit seed extract was compared to the organism. #p < 0.05 when the effects of neutral grapefruit seed and crude grapefruit seed extract were compared. **p < 0.005 when the effect of crude grapefruit extract was compared to the organism.

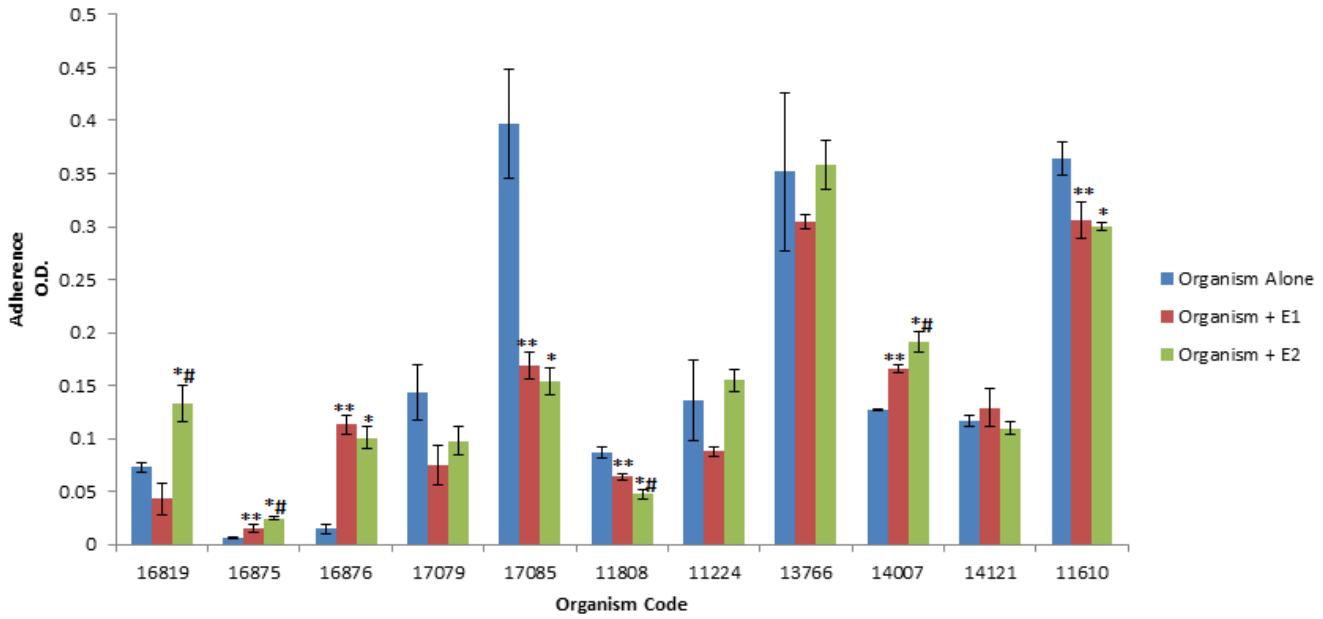

Figure 3. Anti-adhesion effect of 1.03 mg/mL grapefruit juice on bacterial isolates from Mid-stream urine.
E1 = crude grapefruit seed extract with pH 3.8; E2 = neutralized seed extract with pH 7.1. The bars represent mean of three data points and SD (Mean ± SD). P < 0.05 represents statistical differences between treated and untreated groups (one-way ANOVA followed by Tukey's pairwise multiple comparison). *p < 0.05 when the effect of neutral grapefruit seed extract was compared to the organism. #p < 0.05 when the effects of neutral grapefruit seed and crude grapefruit seed extract were compared. **p < 0.005 when the effect of crude grapefruit extract was compared to the organism.

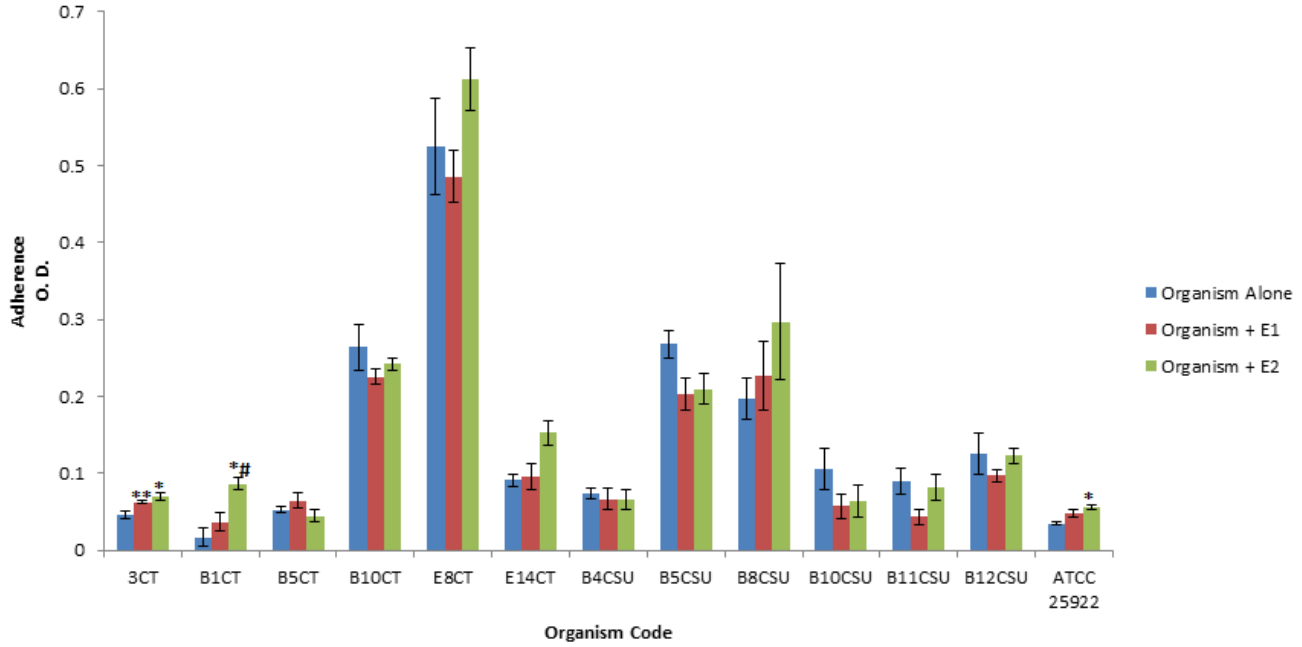

Figure 4. Anti-adhesion effect of 1.03 mg/mL grapefruit juice on bacterial isolates from catheter-stream urine (CSU) and catheter tips (CT).
E1 = crude grapefruit seed extract with pH 3.8; E2 = neutralized seed extract with pH 7.1. The bars represent mean of three data points and SD (Mean ± SD). P < 0.05 represents statistical differences between treated and untreated groups (one-way ANOVA followed by Tukey's pairwise multiple comparison). *p < 0.05 when the effect of neutral grapefruit seed extract was compared to the organism. #p < 0.05 when the effects of neutral grapefruit seed and crude grapefruit seed extract were compared. **p < 0.005 when the effect of crude grapefruit extract was compared to the organism.

However, the intrinsic antibacterial activity and modulatory effect of bergamottin has been investigated and reported (Abulrob et al., 2004). The preliminary data suggested that the grapefruit component (bergamottin epoxide) at sub-inhibitory concentrations enhanced the susceptibility of test methicillin-resistant *Staphylococcus aureus* strains to agents to which the organisms were normally resistant. This has been attributed to efflux pump inhibition capacity of bergamottin, which may explain increased activity of ciprofloxacin when combined with grapefruit seed extract and/or juice in this study.

One of the problems of nalidixic acid is the poor cellular penetration due to its hydrophobicity. The observed increase in its activity when combined with either grapefruit seed or juice may be due to increased cellular permeability to nalidixic acid brought about by the components of grapefruit. Streptomycin, an aminoglycoside, acts by inhibiting protein synthesis. It is possible that the ability of streptomycin to inhibit enzymes involved in protein synthesis might have been potentiated in the bacterial cell by the components of grapefruit. The ability of grapefruit components to inhibit the drug metabolizing enzyme isoform CYP3A4 and CYP3A4 has been reported (Veronese et al., 2003).

Decreased activity exemplified by MICs increase exhibited more at higher concentration of grapefruit seed extract and higher dilution of grapefruit juice can be attributed to competition for the same target sites by both the antibiotics and the extract or juice and selective uptake of extract or juice by the organism.

Bacterial adherence to mucosal cells is an important step in the development of infection (Beachey, 1981). This has been amply demonstrated, especially for urinary tract infections (Kunin, 1987; Reid and Sobel, 1987). Similarly, bacterial adhesion is the first step in the formation of biofilm. Biofilm formation has played role in the persistence of infections due to increased resistance to antimicrobial agents. Resistance to antibiotics by biofilm can be through trapping of antimicrobial agent by exopolysaccharide matrix of biofilm; quorum sensing signalling as well as reduced metabolic process by biofilm. In the face of increasing incidence of antimicrobial resistance and recalcitrance of bacteria to current antimicrobial therapy, drug discovery research is focusing on limiting the pathogenicity mechanisms demonstrated by bacteria through biofilm formation. It therefore follows that agent that will prevent bacterial adhesion to surfaces can be useful in preventing biofilm formation. In this study, the result of anti-adhesion activity of grapefruit seed extract and juice varied. While some bacterial species were able to utilize grapefruit seed extract and/or juice to promote their adhesion, adhesion of some species were significantly reduced at the test concentrations, although there was no total prevention of adhesion in the study. Anti-adhesion activity of both the grapefruit seed extract and juice was more on isolates from the midstream urine (27% vs 36%) and catheter stream urine (28% vs 57%) than on isolates from catheter tips (16% vs 33%). However, grapefruit juice displayed more anti-adhesion activity than grapefruit seed extract. This suggests the presence of components that interfere with bacterial adhesion to surface at higher concentration in juice than in seed extract. The purified limonoids and flavonoids (narigenin) components of citrus have demonstrated significant inhibition of autoinducer-mediated cell–cell signalling and biofilm formation (Girennavar et al., 2008; Vikram et al., 2010; 2011).

Moreover, the anti-adhesion activity of both the grapefruit seed extract and juice was found to be pH dependent as some isolates have their adhesion property restored and enhanced at neutral pH of both the seed extract and juice. Effect of pH on adhesion has previously been reported (Oliveira et al., 1994; Garret et al., 2008).

CONCLUSIONS

The findings in this study suggest that both the grapefruit seed extract and juice possess ability to modulate antibiotic activity and prevent bacterial adhesion. The findings also suggests that a number of mechanisms in combination could be responsible for the reported effectiveness of grapefruit seed extract and juice in the treatment of UTIs. These mechanisms include their inhibitory effects, the antibiotic modulatory activity, which increased activity of some antibiotics, and interference with the adhesion of bacterial isolates by grapefruit components.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The roles played by the duo of Professor Onipede AO and Professor Badmus TA of the Department of Medical Microbiology and Parasitology and Urology unit of Obafemi Awolowo University, Ile-Ife respectively were appreciated.

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