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Antibacterial potential of fresh fruit juices against multi-drug resistant pathogens

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ABSTRACT

Objective: To explore the antibacterial activity of fresh fruit juices against drug-resistant pathogens.

Methods: Fresh juices were prepared by squeezing 7 fruits [*Citrus sinensis*, *Citrus aurantifolia* (*C. aurantifolia*), *Punica granatum* (*P. granatum*), *Malus domestica*, *Ananas comosus*, *Fragaria ananasia*, and *Actinidia deliciosa*]. The antibacterial activities were studied by using well-diffusion, minimum inhibitory (MIC), and minimum bactericidal (MBC) assays against 7 clinical pathogens: Gram-positive, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*; Gram-negative, 2 strains of *Pseudomonas aeruginosa*, 2 strains of *Klebsiella pneumoniae*.

Results: The diffusion test revealed that *Malus domestica* juice had no antibacterial activity against the tested pathogens; *Citrus sinensis* and *Ananas comosus* revealed low antibacterial activity; *Fragaria ananasia* and *Actinidia deliciosa* revealed moderate antibacterial activity; *P. granatum* and *C. aurantifolia* exhibited high antibacterial activity against most of the clinical strains. MIC and MBC tests exhibited that *P. granatum* and *C. aurantifolia* had noticeable bactericidal effects with MBC/MIC values ranging between 2 to 4.

Conclusions: The crude fresh juices of *P. gronatum* and *C. aurantifolia* have potential as natural therapeutic agents against some multi-drug resistant bacteria and they could prevent pathogenic diseases.

KEYWORDS: MDR pathogens; Fruits; Antibacterial; Cup-plate diffusion; MIC; MBC

1. Introduction

For centuries, we benefit from fruits in food and health purposes. The World health organization recommended the consumption of at least 400 g per capita per day for prevention from chronic diseases[1] and unfortunately, yet the global rate of intake per capita is lower than dietary recommendations[2]. The concept of "nutraceutical" has gradually become popular among people all over the world. Nutraceuticals are dietary supplements of processed food that help improve health and prevent diseases[3]. Fruits are considered as nutraceutical products because they contain various bio-active agents such as polyphenols that can promote human health[4]. Researches have proven the nutritional values of edible fruits and the consumption of fruits as protection against many chronic diseases such as, cardiovascular diseases, obesity, and metabolic syndrome abnormalities[5], and it is also reported that some fruits could reduce the risk of many cancers[6], but average body weight and diabetes have yet to be thought about[7].

Commercial fruits were rich in phytochemical compounds such as phenolic compounds, terpenes, and carotenoids[8]. *Malus domestica* (*M. domestica*) is rich in antioxidant compounds such as quercetin, chlorogenic acid, catechin, and phloridzin, and it could reduce the risk of lung cancer and coronary heart disease[9]. *Citrus sinensis* (*C. sinensis*) are rich in vitamin C, polyphenols, hesperidin flavonoids, limonoids, synephrine, and pectin, and is a distinguished antioxidant and immune system stimulator[10]. *Ananas comosus* (*A. comosus*) the third important tropical fruits in the world, is rich in minerals, vitamins, bromelain, and malic acid and has antioxidant, anti-inflammatory and digestion promoting activities[11]. *Fragaria ananasia* (*F. ananasia*) is rich in nutrients, vitamins, and phytochemicals such as anthocyanins, phenolics, and flavonoids, and has anti-inflammatory, antioxidant, and

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antihypertensive potential[12]. *Punica granatum* (*P. granatum*) is rich in phenolic compounds, flavonoids, pro-antho-cyanidin compounds, and ellagitannins. Studies also confirmed its anti-cancer, anti-cardiovascular disorders, anti-osteoporotic, anti-estrogenic, antioxidant and antimicrobial activity[13]. *Actinidia deliciosa* (*A. deliciosa*) is rich in dietary fibers, vitamins, and folate, and can enhance the gastrointestinal functions and modulate the glycemic responses as a good antioxidant agent[14]. *Citrus aurantifolia* (*C. aurantifolia*) is also rich in phytochemicals like phenolic compounds, flavonoids, carotenoids, and limonoids, and could be used as antimicrobial, diuretic, anthelmintic, astringent, and mosquito bites repellent[15].

Nowadays, the efficacy of antibiotics is not steady. Due to the emergence of multi-drug resistant (MDR) strains, it needs the development of new antibiotics[16-18]. Scientists suggested some alternatives to conventional antibiotics, including vaccines, antibodies, engineered bacteriophages, and immune stimulation[19]. Bioactive constituents from natural products and medicinal plants could also serve as a viable alternative[20]. Many researchers have studied on secondary metabolites from plants, hoping to discover new antimicrobial agents.

However, the relationship between fresh fruit juices and health is still controversial and more investigations are required[8,21]. The antibacterial potential of the fresh fruit juices is not well investigated neither. Therefore, the current study explores the antibacterial activity (*in vitro*) of crude fresh juices from *C. sinensis*, C. *aurantifolia*, *P. gronatum*, *M. domestica*, *A. comosus*, *F. ananasia*, and *A. deliciosa* against seven strains of human pathogens.

2. Materials and methods

2.1. Microorganisms

Bacterial pathogens were maintained at 5 ℃ in Petri-dishes containing Muller-Hinton agar, which were previously identified by the VITEK 2 automated system. These identified clinical isolates were generously provided by Dr. Alqadi from the Department of Pathology and Laboratory Medicine, ArRass General Hospital, KSA. The isolates included 3 Gram-positive strains: *Staphylococcus aureus* (*S. aureus*) (from abscess), methicillin-resistant *S. aureus* (MRSA) (from blood), *Staphylococcus epidermidis* (from wound swab), and 4 Gram-negative strains: *Pseudomonas aeruginosa* 1 (*P. aeruginosa* 1) (from pus), *P. aeruginosa* 2 (from wound swab), *Klebsiella pneumoniae* 1 (*K. pneumoniae* 1) (from sputum) and *K. pneumoniae* 2 (from wound swab).

2.2.Plant materials and juicing

A total of 7 fruits were selected and the details about these fruits are presented in Supplementary Table 1. We chose crude fresh juices to increase patients' acceptance and avoid toxicity. Fresh fruits were purchased from local markets in Qassim, Saudi Arabia and washed. The processing was performed under aseptic conditions. The fruits were squeezed and turned into juice by using a fruit juice machine (without adding water) (Supplementary Figure 1).

2.3.Antibiotics susceptibility testing

The antibacterial susceptibilities of 7 clinical isolates were determined by Kirby-Bauer's disc diffusion method following the Clinical and Laboratory Standards Institute guidelines[22] with minor modifications. Briefly, after overnight culture, an inoculum was adjusted to 0.5 McFarland standard and added into nutrient agar plates. Antibiotics rings were loaded (Mastering-S^{TM}, UK). Plates were then incubated overnight for 18-24 h. The growth of inhibition zones was recorded. The following antibiotics were used: penicillin g (10 units/disk), augmentin (30 μg/disk), clindamycin (2 μg/disk), cefoxitin (30 μg/disk), metronidazole (5 μg/disk), piperacillin (100 μg/disk), imipenem 10 μg/disk), ceftazidime (30 μg/disk), ciproflozacin (5 μg/disk), aztreonam (30 μg/disk) and tobramycin (10 μg/disk).

2.4. Antibacterial activity screening

The antibacterial activity was tested (*in vitro*) using standard agar well diffusion assay[23]. Briefly, Petri-dishes were prepared by loading about 25 mL of an autoclaved nutrient agar on sterile plates and left to solidify. Then, the surface of each plate was drilled using a sterile cork-borer (6 mm) and 4 wells were punched out on each plate. A total of 100 μL of a standardized culture (adjusted to 0.5 McFarland ≈ 107 CFU/mL) of test organisms was added into the agar plate followed by loading of 50 μL of the crude fresh juices in the wells, and then 50 μL of chloramphenicol (2.5 mg/mL) was also loaded to other wells and served as a positive control. The seeded Petri-dishes were incubated for 24 h at 37 ℃ and then the zone of inhibition was recorded. The mean zone of inhibition was calculated based on 4 replicates. Based on Clinical and Laboratory Standards Institute guidelines, the zone of inhibition less than 10 mm considered as a weak activity, above 10 and less than 13 mm considered as a moderate activity and from 13 mm and above considered as a high antibacterial activity[24,25].

2.5. Determination of minimum inhibitory concentration (MIC)

The fruit juices with remarkable antibacterial activity by the well-diffusion assay were subjected to MIC test[26]. A toal of 1 mL of Mueller-Hinton broth was poured to a set of 7 test tubes and autoclaved. Subsequently, 1 mL of 100% fresh fruit juice was poured to the 1st test tube to make a concentration of 50%, and twofold serial dilutions were made by transferring 1 mL from one tube to another to get the following series: 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, and 0.78%. Then, 100 μL/mL of the standardized bacterial culture was loaded to each tube. Negative control was made by pouring 1 mL of physiological saline instead of fruit juice. The lowest concentration of the dilution without bacterial growth was considered as MIC.

2.6. Determination of the minimum bactericidal concentration (MBC)

MBC values were determined after the MIC test[27]. Briefly, 50 μL was taken by the Eppendorf pipette from each MIC tubes and spotted over nutrient agar plates, which were then incubated overnight at 30℃ -35℃. The lowest MIC that revealed no visible growth was regarded as MBC. The MBC/MIC was also calculated as bactericidal or bacteriostatic.

2.7. Statistical analysis

SPSS software 14.0 (SPSS Inc., Chicago, USA) was utilized for statistical analysis. Data were expressed as means±standard deviation. Measurement data with normal distribution were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* test (Tukey HSD) with a significance level of α=0.05.

3. Results

3.1. Susceptibility results

The susceptibility results are presented in Table 1. *S. aureus*, MRSA, *Staphylococcus epidermidis*, *P. aeruginosa* 1, *P. aeruginosa* 2, *K. pneumoniae* 1, and *K. pneumoniae* 2 were resistant to 3, 5, 3, 5, 6, 7 and 4 types of antibiotics, with resistance rate 27.2%, 45.5%, 27.2%, 45.5%, 54.5%, 63.6%, and 36.4%, respectively. The Gram-negative isolates showed higher resistance rates.

3.2. Result of well-diffusion test

The well-diffusion test showed that all fruit juices had antibacterial effects, except *M. domestica* (Supplementary Figure 2 and Supplementary Table 2). *C. sinensis* and *A. comosus* showed weak activity; *F. ananasia* and *A. deliciosa* showed moderate activity; *C. aurantifolia* and *P. granatum* showed high activity against most of the clinical strains. The antibacterial activities of the juices of *C. aurantifolia* and *P. granatum* were significantly higher than other juices ($P<0.05$). However, the zones of inhibitionms made by juices of *C. aurantifolia* and *P. granatum* were lower than the standard drug (Figure 1).

3.3. MIC and MBC

Fruit juices with high antibacterial activity in the well-diffusion test (≤13.0 mm) were determined further in MIC and MBC tests. The MIC and MBC values of *C. aurantifolia* and *P. granatum* are shown in Table 2. The MIC and the MBC of *C. aurantifolia* against MRSA (bacteriostatic) were 3.12% and 6.25%, respectively. While, the MIC and MBC values against other tested strains were 6.25% and 12.50%, respectively. For *P. granatum*, the most

Table 1. Antibiotics sensitivity patterns of the tested organisms.

Clinical isolates	Antibiotics											Resistance percentage (%)
	PG	AUG	CD	FOX	MZ	PRL	IMI	CAZ	CIP	ATM	TN	
Sa	R	S	S	S	R	R	S	S	S	S	S	27.2
MRSA	R	S	S	S	R	S	R	R	S	R	S	45.5
Se	R	S	S	S	R	S	R	S	S	S	S	27.2
Pa1	R	R	R	R	R	S	S	S	S	S	S	45.5
Pa2	R	R	R	R	R	R	S	S	S	S	S	54.5
Kp1	R	R	R	R	R	R	S	R	S	S	S	63.6
Kp2	R	S	R	S	R	R	S	S	S	S	S	36.4

Sa: *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*; Se: *Staphylococcus epidermidis*; Pa1: *Pseudomonas aeruginosa* (from pus); Pa2: *Pseudomonas aeruginosa* (from wound swab); Kp1: *Klebsiella pneumoniae* (from sputum); Kp2: *Klebsiella pneumoniae* (from wound swab). PG: Penicillin G; AUG: Augmentin; CD: Clindamycin; FOX: Cefoxitin; MZ: Metronidazole; PRL: Piperacillin; IMI: Imipenem; CAZ: Ceftazidime, CIP: Ciproflozacin; ATM: Aztreonam; TN: Tobramycin.

Table 2. MIC and MBC values.

Clinical strains	Fresh juice of *Citrus aurantifolia*			Fresh juice of *Ananas comosus*		
	MIC (%)	MBC (%)	MBC/MIC	MIC (%)	MBC (%)	MBC/MIC
Sa	6.25	12.50	2	12.50	50.00	4
MRSA	3.12	6.25	2	3.12	6.25	2
Se	12.50	12.50	1	12.50	50.00	4
Pa1	6.25	12.50	2	6.25	25.00	4
Pa2	6.25	12.50	2	12.50	50.00	4
Kp1	6.25	12.50	2	12.50	50.00	4
Kp2	6.25	12.50	2	12.50	50.00	4

Sa: *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*; Se: *Staphylococcus epidermidis*; Pa1: *Pseudomonas aeruginosa* (from pus); Pa2: *Pseudomonas aeruginosa* (from wound swab); Kp1: *Klebsiella pneumoniae* (from sputum); Kp2: *Klebsiella pneumoniae* (from wound swab); MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

susceptible bacterial strains was MRSA, with MIC and MBC as 3.12% and 6.25%, respectively, followed by *P. aeruginosa* (from pus), with MIC and MBC as 6.25% and 25.00%, respectively. The MIC and MBC against the other tested strains was 12.5% and 50.00%, respectively. The MBC/MIC value ranged between 1 to 2 for *C. aurantifolia* and 2 to 4 for *A. comosus*.

4. Discussion

The present study reports the antibacterial properties of seven fresh fruit juices against seven clinical strains. The microorganisms were randomly selected and the antibiotics profile showed that they could be considered as MDR bacteria based on the definition that MDR bacteria are resistant (*in vitro*) to three or more antibacterial classes[28]. Our study showed that the Gram-negatives were highly resistant. It is similar to previous reports, which stated that due to its peptidoglycan layer, the Gram-negative bacteria are more resistant to regular antibiotics especially some nosocomial strains such as *P. aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii*[29,30].

Our findings showed that *M. domestica* had no antibacterial activity against MDR strains; *C. sinensis* and *A. comosus* exhibited weak activities; *F. ananasia* and *A. deliciosa* revealed moderate activity; *C. aurantifolia* and *P. granatum* showed high activity against most of the clinical strains. We also found that some

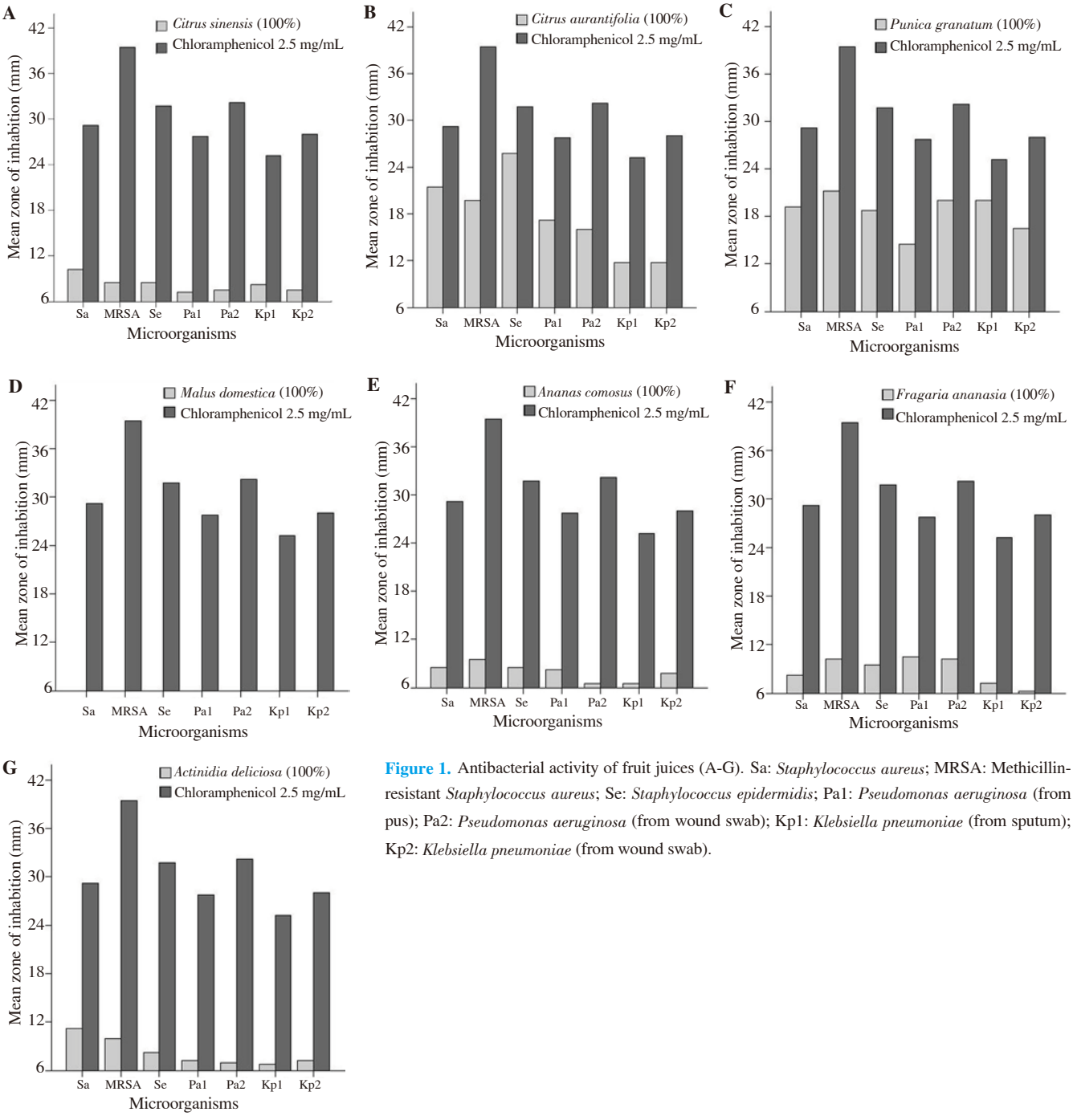

Figure 1. Antibacterial activity of fruit juices (A-G). Sa: *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*; Se: *Staphylococcus epidermidis*; Pa1: *Pseudomonas aeruginosa* (from pus); Pa2: *Pseudomonas aeruginosa* (from wound swab); Kp1: *Klebsiella pneumoniae* (from sputum); Kp2: *Klebsiella pneumoniae* (from wound swab).

pathogens showed moderate susceptibility to some "moderate effectiveness" fruit juices. These results prove fresh fruit juices as natural antibacterial agents. Some previous studies have investigated the antimicrobial properties of fresh juices against various microorganisms; however, little evaluated the effects on MDR pathogens. It is reported that *P. granatum* fresh juice showed the highest antimicrobial activity, followed by *Psidium guajava* and the least antibacterial activity was from *M. domestica* fresh juice[31]. Another study tested the antibacterial activity of 3 fruits against urinary tract infection bacteria, and found that *M. domestica* and *Carica papaya* did not show any inhibition zones, while *Citrus sp* and *F. ananasia* showed remarkable antibacterial activity[32]. It is reported that *Citrus limon* has significant antibacterial activity against diarrhea-causing pathogens[33].

Moreover, we also found that *M. domestica*, *C. sinensis*, *A. comosus*, *F. ananasia*, and *A. deliciosa* exhibited no activity, weak, and moderate antibacterial properties, respectively; while in some previous studies they were reported as good antimicrobial agents. That may be attributed to that most studies used extracts or specific phytochemicals such as phenolics and flavonoids, whereas we tested 100% crude juice. More than 80% the juice is water and it may lead to dilution of antibacterial constituents. In addition, we mostly used the fruit pulp, whereas, many positive studies used the peels of the fruits. Referring to these observations, it was reported that the polyphenols fraction extracted from the peel of *Malus spp.* displayed noticeable antibacterial activity against different Gram-positive and Gram-negative bacteria[34]. Ethanol extract of *A. comosus* showed good antibacterial activity against MDR *P. aeruginosa*[35]; The methanol extract of the peel of the *C. sinensis* had marked antibacterial activity against *K. pneumoniae* and *Bacillus cereus*[36].

The fruit juices with high antibacterial activity against most pathogens were investigated for the MIC and MBC in this study. As known, MIC or MBC are the lowest concentration of an antibacterial agent necessary to inhibit bacterial growth or kill bacteria, repectively. Accordingly, *P. granatum* exhibited low MIC and MBC values, while, *C. aurantifolia* showed lower MIC and MBC values. So we calculate MBC/MIC, where the tested compounds are deemed as bactericidal if the values of MBC/MIC $\leqslant$4 and deemed as bacteriostatic if MBC/MIC$>$4[37]. Although, *P. granatum* juice showed bactericidal effects (MBC/MIC values ranging from 2 to 4), *C. aurantifolia* juice had more noticeable bactericidal activity (MBC/MIC values ranging from 1 to 2), which require further investigations to isolate these antibacterial constituents. The findings are in consistent with some previous studies. The essential oil of *C. aurantifolia*, which was rich in limonene, inhibited the growth of all tested Gram-negative and Gram-positive bacterial pathogens isolated from fishes and showed some bactericidal effects (MBC/MIC=2 to 8)[38]. It was reported that *P. granatum* fresh juice was rich in polyphenols and at a concentration of 20% and showed a MIC ratio of 100% (at concentration 20%) against 60 bacterial strains[39]. Furthermore, our study indicated that fresh juices of *C. aurantifolia* and *P. granatum* have a considerable bactericidal effect against MRSA. Based on the above, fresh juices of *C. aurantifolia* and *P. granatum* are recommended as natural antibacterial beverages and are recommended to patients who are not allergic to these fruits. However, *in vivo* pharmacological studies are required to determine the effective dosage and possible therapeutic complications.

5. Conclusion

The increasing prevalence of MDR bacteria is a major concern worldwide, which requires cooperation among medical, biological, and nutritional sciences. This investigation highlights the significant antibacterial activity of some fruit fresh juices against MDR pathogens. *C. aurantifolia* and *P. granatum* are good candidates for natural antibacterial agents. The study also supports the possibility of introducing such fruit juices to the diet of patients and medical personnel in order to control the spread of MDR pathogens and the nosocomial infections in hospitals.

Conflict of interest statement

The author reports no conflict of interest.

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Authors' contribution

E.M.A. (the sole author) has conceptualized, designed, analyzed, interpreted, prepared and edited the entire manuscript.

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