RESEARCH ARTICLE

Ellagic acid-rich Pomegranate extracts synergizes moxifloxacin against methicillin resistance *Staphylococcus aureus* (MRSA)

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Abstract

Objective: To elucidate the anti- Methicillin resistance Staphylococcus aureus (MRSA) effect of pomegranate alone and in combination with moxifloxacin fluoroquinolone.

Methods: A total of five clinical isolates of MRSA (ATCC 43300) were used in the study. Disc diffusion method was used to determine the anti-MRSA effect of pomegranate and/or moxifloxacin by using Mueller-Hinton agar. Minimal inhibitory concentration (MIC), fractional inhibitory concentration (FIC) of moxifloxacin and pomegranate were calculated, the dynamic picture of the bactericidal effect of pomegranate and/or moxifloxacin was determined. SPSS version 20.00 was used for data analysis.

Results: Zone of inhibition (ZOI) of moxifloxacin was 19.67±4.84mm which was not significant compared with pomegranate ZOI 14.59±2.73mm, (P=0.07). The combination of moxifloxacin and pomegranate led to more significant ZOI (26.83±4.91mm) compared with moxifloxacin (P=0.04) and pomegranate alone (P=0.0012). MIC of pomegranate was high (31.62±6.95µg/mL) compared with low MIC of moxifloxacin (2.70±0.63 µg/mL), (P<0.0001), while MIC of the combination was low (1.22±0.85µg/mL) compared MIC of moxifloxacin, (P=0.012). FIC of pomegranate was 0.038 and FIC of moxifloxacin was 0.45, therefore ΣFIC index was 0.488, equal to the synergistic effect. The kill rates of combination were higher than those of pomegranate or moxifloxacin, against MRSA ATCC 43300.

Conclusion: Ellagic acid-rich pomegranate extract alone has significant antibacterial activity and synergizes the bactericidal effect of moxifloxacin against MRSA.

Keywords: Methicilin Resistance *Staphylococcus Aureus* (MRSA), Pomegranate, Moxifloxacin.

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Introduction

There is an urgent and incessant need to discover and find out new antimicrobial compounds with novel mechanisms to combat the alarming bacterial resistance against conventional used antibiotics. There are different strategies to control and alleviate the infections caused by multi- antibiotic resistance strains of bacteria, one of which is through isolation of different phytochemicals from different herbs, which could control the spread of infections.[1] In a similar way, synergistic combinations using different phytochemicals and commercially available antibiotics are an effective method against resistance bacteria and regarded as potential resistance modifying agents.[2] The latent mechanism of phytochemicals in the potentiating of antibiotic effects is through inhibition of antibiotic efflux or synergizing the antibiotic effects at the site of actions.[3] In addition, the extensive use of antibiotics in the treatment of bacterial infections had led to the emergence and appearance of resistant bacterial strains.

Staphylococcus aureus (*S. aureus*) is one of the most resistant bacteria associated with health care and community associated infections.[4] *S. aureus* is a commensal bacteria, which is frequently present on the skin and nasal mucous membrane, as 20% of individuals are persistent carriers of *S. aureus*. Historically, *S. aureus* resistance was emerged within two years of penicillin discovery in 1942, after that, methicillin was introduced as an alternative in the late 1950 until methicillin resistance to *Staphylococcus aureus* (MRSA) was developed in 1960.[5,6] Therefore, MRSA is resistant to most antibiotics and efforts to find phytochemicals alone or in combination with different antibiotics have been extensively studied.

The pomegranate (Punica granatum L.) has an extended and long history of antibacterial use dating to Egyptian, biblical times, against diarrhoea and dysentery. With time, different studies have been conducted for testing the

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potential antibacterial activity and mechanism of pomegranate.[7] These studies determined that different extracts of pomegranate have different antibacterial potency as methanol extracts illustrate wide antibacterial activity compared with aqueous extracts.[8] Voravuthikunchai and Kitpipit[9] found that pomegranates ethanoic extracts have noteworthy antibacterial activity against *S. aureus* and MRSA. Similarly, Su et al.,[10] reported that pomegranate polyphenols reduce in vitro multiplication of MRSA at a concentration of 0.2-0.4mg/mL within 24 hours.

Therefore, objective of the present study was to observe the anti-MRAS effect of pomegranate alone and in combination with moxifloxacin fluoroquinolone antibiotic.

Materials and Methods

The selected bacteria: A total five clinical isolates of MRSA (ATCC 43300) were used in this study purchased from National Microbiology Institutes, College of Science, Baghdad University. Bacterial strains were incubated and maintained in nutrient agars at 37°C for 24 hours and one colony from MRSA stock culture was inoculated in 4mL of nutrient broth.

Chemicals and drugs: Moxifloxacin 400mg tablet (MAHAFLOX 400mg, mankind Pharma, Ltd, India) and pomegranate extract 1g capsule (pomegranate seed extract 70% ellagic acid, CA92614, USA) were purchased from the private pharmaceutical company. Stock solutions of antibiotic and pomegranate were prepared and dilutions made according to the clinical and laboratory institute (CLSI) guidelines.[11] Dimethyl-sulfoxide (DMSO) was used as a solvent and had no any effect against MRSA and regarded as negative control.

Determination of antibacterial activity

Measurement the inhibition of MRSA growth: Disc diffusion method was used to determine the anti-MRSA effect of pomegranate and/or moxifloxacin by using Mueller-Hinton agar[12] which was poured in 9cm sterile petri dishes. 6mm paper discs contain pomegranate, moxifloxacin or their combination were prepared and placed against DMSO on the inculcated agar surface. Petri-dishes were incubated at 37°C for 24h. Zone of inhibition (ZOI) which represents the clear area around the impregnated disc was determined through measuring the diameter of ZOI. The dimension of ZOI was regarded as an indicator of the effectiveness of pomegranate, moxifloxacin and their combination. If the edge of ZOI was regular, the diameter represented the ZOI whereas; if the edge of ZOI was irregular, radius of ZOI and multiplied by 2 represented the ZOI.

Measurement of minimal inhibitory concentration: According to the National Committee for Clinical Laboratory Standard (NCCLS),[13] the serial dilution method was used to obtain the stock solution of 100mg of moxifloxacin and 100mg pomegranates that were dissolved in 1mL of DMSO with subsequent dilutions. The serially diluted solutions containing pomegranate, moxifloxacin or their combination were added to plates containing Mueller-Hinton agar. Steer replicator containing 5×105CFU/drop of MRSA was placed on each prepared plates and incubated at 37°C for 18h. Minimal inhibitory concentration (MIC) represents and symbolized the lowest concentration of used agent at which no visible bacterial growth was detected. Plates were read in duplicate, and MICs were recorded according to the breakpoints tables of NCCLS to conclude susceptibility or resistance of MRSA to the selected agent and antibiotic.

Determination of synergy test

Fractional inhibitory concentration: 0.5 McFarland turbidity standard of MRSA isolates were inculcated on Mueller-Hinton agar plates and impregnated moxifloxacin disc alone or in combination pomegranate was placed onto each plate that were incubated for 48h at 37°C. For testing the combination, moxifloxacin disc was placed for 1h, it was replaced with a pomegranate impregnated disc on the same agar and vice versa for pomegranate. From these calculated MICs, fractional inhibitory concentration (FIC) of moxifloxacin and pomegranate were calculated.

FIC of moxifloxacin

$$(A) = \frac{\text{MIC of A in combination}}{\text{MIC of A alone}}$$

FIC of pomegranate

$$(B) = \frac{\text{MIC of B in combination}}{\text{MIC of B alone}}$$

FIC index= FIC (A) +FIC (B). Therefore, ΣFIC<0.5 indicate a synergy, ΣFIC 0.5-4.0 indicate an indifference, and ΣFIC >4.0 indicate an antagonism.[14]

Time-kill assay: The dynamic picture of the bactericidal effect of pomegranate and/or moxifloxacin was determined by NCCLS. MRSA isolates were incubated with pomegranate and/or moxifloxacin at 37°C, and viable bacterial counts (CFU/mL) were done after 0, 4, 8 and, 24 h. 1/4 of MIC of the tested agents for each isolates. This curve was plotted as log (CFU/mL) over 24h; synergism is equal to the reduction of colony count more than 100 CFU/mL, antagonism is equal to the increase of colony count more than 100 CFU/mL.[15]

Data analysis was done by using SPSS (IBM SPSS Statistics for Windows version 20.0, 2014 Armonk, NY, IBM, Corp). The data was presented as mean± SD and unpaired student t test was used to determine the level of differences. Analysis of variance (ANOVA) followed by Benferroni post-hoc test was used to compare results of study variables among different groups. The level of significance was regarded when P<0.05.

Results

Zone of inhibition (ZOI): In vitro results, illustrated that moxifloxacin, pomegranate and their combination were effective against MRSA compared with the negative control P<0.0001. ZOI of moxifloxacin was 19.67±4.84mm, which was not significant compared with pomegranate ZOI 14.59±2.73mm, (P=0.07). The combination of moxifloxacin and pomegranate observed more significant ZOI (26.83±4.91mm) compared with moxifloxacin (P=0.04) or pomegranate alone (P=0.0012), (Figure-1).

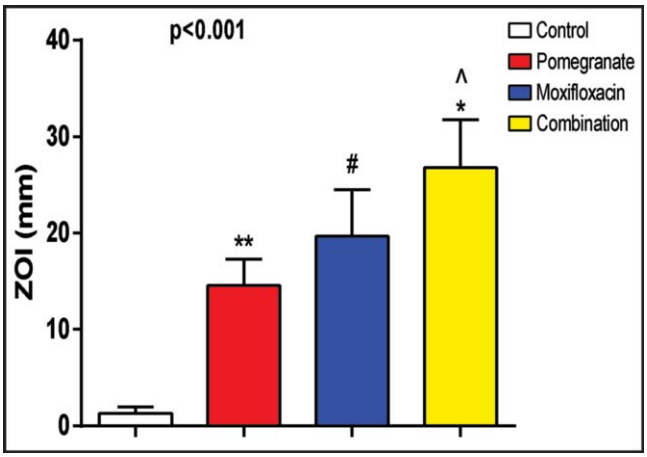

Figure-1: Effects of pomegranate and/or moxifloxacin on the growth of MRSA, illustrated by zone of inhibition (ZOI).

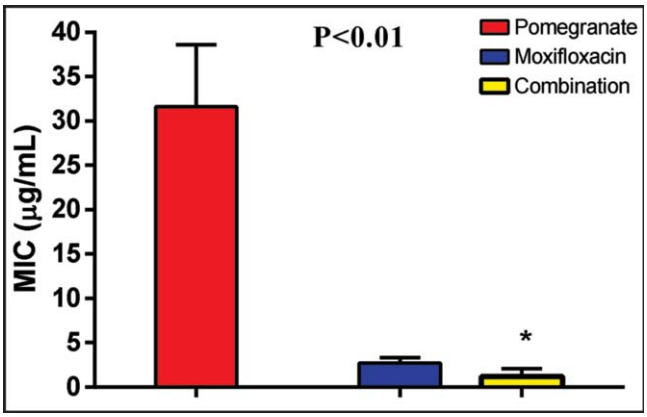

Figure-2: Minimal inhibitory concentration (MIC) of pomegranate and/or moxifloxacin on MRSA.

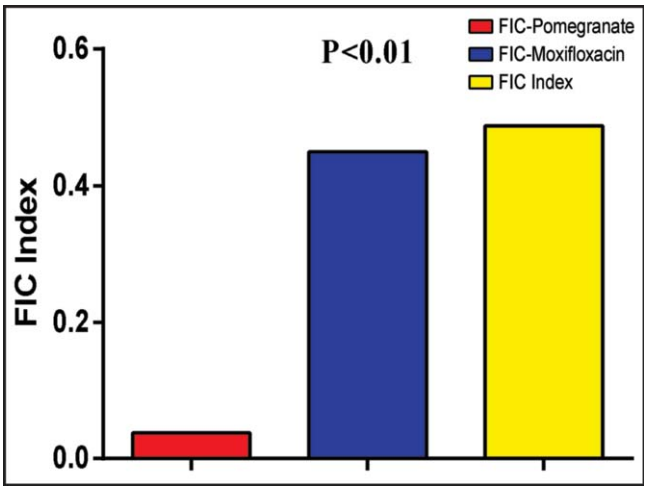

Figure-3: Fractional inhibitory concentration of pomegranate and moxifloxacin on MRSA.

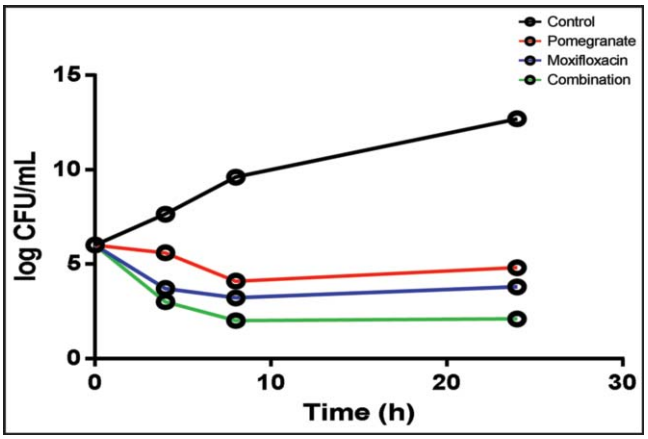

Figure-4: Time-kill curves of pomegranate and/or moxifloxacin at1/4 of MICs against MRSA.

Minimal inhibitory concentration (MIC): The MIC of the pomegranate was higher (31.62±6.95µg/mL) compared with low MIC of moxifloxacin (2.70±0.63 µg/mL), P<0.0001, while MIC of the combination was lower (1.22±0.85 µg/mL) compared MIC of moxifloxacin, P=0.012, (Figure-2).

Fractional inhibitory concentration (FIC): The FIC of the pomegranate was 0.038 and FIC of moxifloxacin was 0.45, therefore ΣFIC index was 0.488, equal to the synergistic effect, (Figure-3).

Bactericidal and time-kill assay: Time-kill assay and bactericidal effect of pomegranate, moxifloxacin, and their combination of one-quarter the MIC for the MRSA ATCC 43300 resulted in synergism with a superior effect at the first 4 hours. The kill rates of combination were higher

than those of pomegranate or moxifloxacin alone, against MRSA ATCC 43300, (Figure-4).

Discussion

MRSA is the main cause of nosocomial infections due to resistance to the commonly used antibiotics and other antimicrobial agents like penicillin, cephalosporin, tetracycline, chloramphenicol and macrolides. Therefore, this bacterium is regarded as a challenge to the existence antimicrobial agents.[16] For that reason, continued searches were and are done to identify new therapeutic agents with noteworthy efficacy against MRSA.

The results of the present study illustrated that pomegranate led to significant antibacterial activity against MRSA compared to negative and positive controls as showed by different previous studies.[17,18] Findings of the present study showed that pomegranate ZOI against MRSA was not significantly different from moxifloxacin which was used as a positive control. This verdict gives a clue that pomegranate demonstrated anti-MRSA effects similar to that of moxifloxacin. Bindu et al[19] showed that pomegranate leads to significant inhibition of the growth of MRSA with 21mm ZOI, but in our study ZOI was equal to 14.59±2.73mm. Also, the MIC of pomegranate of the present study was 31.62±6.95µg/mL compared with high pomegranate MIC of Bindu et al, which was 125µg/ mL. The reasons behind better and good results of the present study was to use the prepared pomegranate seed extracts containing 70% ellagic acid compared to alcoholic or aqueous extracts of pomegranate active constituents which have different antibacterial activity.[20]

It has been reported that pomegranate has significant wide-spectrum antibacterial activity against different Gram-positive and Gram-negative bacteria due to the presence of phyto-constituents such as phenolic and saponin compounds, which are mainly derived from pomegranate bark.[21] In the present study, pomegranate seed extract was used, which contains mainly ellagic acid, a most potent antibacterial constituent as suggested by Rosas-Burgos et al.[22]

On the other hand, pomegranate led to synergistic effect when combined with moxifloxacin as ΣFIC index was less than 0.5 as shown by Hemaiswarya et al[23] who observed the synergistic effect between natural product flavones and different antibiotics. It has been noticed, that ellagic acid had an inhibitory effect on bacterial DNA-gyrase, which might explain the synergism with moxifloxacin, as the latter acts through inhibition of this pathway.[24] Ellagic acid from pomegranate is highly lipophilic and has ability to inhibit MRSA DNA-gyrase and prevents cleavage of DNA during bacterial replication process.[25] Therefore, ellagic acid-rich pomegranate extract leads to the bactericidal effect against MRSA bacteria when used alone or in combination with moxifloxacin, which in part explains the high percentage of the killing rate of this combination as compared with pomegranate or moxifloxacin alone.

Limitations of this study were small sample size of bacterial strains, antibacterial activity of pomegranate against methicillin sensitive *Staphylococcus aureus* (MSSA) was not evaluated, and other constituents also were not evaluated against MRSA. In spite of these limitations, this study is regarded as a novel study concerning the synergistic bactericidal effect of pomegranate and/or moxifloxacin against MRSA.

Conclusion

Ellagic acid-rich pomegranate extracts alone have significant antibacterial activity and synergize the bactericidal effect of moxifloxacin against in vitro MRSA. Therefore, ellagic acid is a promising new agent against refractory infections caused by high resistance and challenged MRSA bacteria.

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Conflict of Interest: None.

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