Antibacterial activity of Pomegranate molasses (alone and in combination with Ampicillin and Ciprofloxacin) on multidrug resistant *Serratia marcescens*

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Abstract. The antibacterial activity of Pomegranate molasses against *Serratia marcescens* bacteria was determined. The correlation between molasses and antibiotics were evaluated by using fractional inhibitory concentration.

1. Introduction

*Serratia marcescens* is gram-negative bacteria and one of the most important bacterial species responsible for different infections in human body. It is opportunistic pathogen in immunocompromised patients because its presence in the air, water, and food as well as the spread in the hospital environment [1]. Many virulence factors were secreted by these bacteria to play important role in increasing their ability to causes disease, such as the hemolysin (which lead to lyses the erythrocytes cells by form holes in its wall) and biofilm which consists of complex made up of viable and dead bacteria cells held within an extracellular matrix made up of polysaccharide, protein and extracellular DNA) [2]. In addition, to the ability of bacteria to form adhesion factors that play an important role in its pathogenicity [3].

The antibiotics are one of the most common methods that use to eliminate pathogenic bacteria and of great benefit in maintaining human health, however, the resistance of many pathogenic bacteria to these antibiotics is one of the biggest challenges facing human as well as causing toxic reactions in humans’ body [4]. Therefore, there is a need to find alternative method to avoid antibiotic resistance problem, one of this method is use the medical plants extracts. Some researchers highlighted the use of medicinal plant extracts as an alternative to antibiotics because of its own ability to improve the immune system of humans and work as the killer or inhibitor of bacterial growth inside the body [5].

Currently, there is considerable interest toward evaluating plant sources for use against multidrug-resistant pathogenic bacteria. Various components found in many types of medicinal plants, some of which can operate in synergy with drugs to kill pathogenic bacteria but others are able to sensitize the pathogen to antibiotic [6].

Pomegranate is one of the most important medicinal plants, which contains many therapeutic compounds. Also, is an important source of phenolic compounds with effective antioxidants on the one hand and anti-bacterial activity on the other hand [7].

The purpose of this study was to determine the safe way to reduce the virulence of pathogenic bacteria by using a new method. The simultaneous use of antibiotics (which began to lose their ability to kill bacteria) with plant extracts as a natural source of some substances active in increasing the sensitivity of bacteria to antibiotics and inhibit their ability to produce some virulence factors.
2. Materials and Methods

2.1 Bacterial isolates
The microbiology lab in the biology department, College of Science, Kerbala University, supplied 30 Serratia marcescens isolates (these isolates were obtained from women with urinary tract infection aged from 26-40 years attending Maternity and Women’s Hospital in Kerbala province). The isolated bacteria were already diagnosed biochemically according to methods described by Collee et al. [8] and Baron et al. [9]. Finally, the diagnosis of isolates bacteria were confirmed by using the API-20E system applied depending on the instructions manufacturer.

2.2 Ability of isolated bacteria to produce virulence factors
As described by Collee et al [8] and Baron et al [9] the ability of isolated bacteria to produce hemolysin and protease enzyme were determined by culture isolates on blood and skim milk agar plates respectively. In addition, the ability of isolated bacteria to form biofilm was determined by tube and tissue culture plat methods as described by Mathur et al [10] and Maldonado et al [11].

2.3 Determination of the minimum inhibitory concentration (MIC) of molasses and antibiotics.
Pomegranate molasses got ready from the local markets of the Kerbala city. The minimum inhibitory concentration (MIC) of the dry pomegranate molasses (the 1g of dry pomegranate molasses were dissolved in 10 ml of distal water to prepare stock solution 100 mg/ml diluted with sterile to prepared gradual concentration ranging from 10-80 mg/ml) and to antibiotic (the 1mg of antibiotic were dissolved in 10 ml of distal water to prepare stock solution 100 µg/ml diluted with sterile to prepared gradual concentration ranging from 10-80 µg/ml) as described by CLSI [13]. The MIC was taken as first dilution that inhibited the growth of the tested bacteria or represented by the last concentration with little or no visible growth. The MIC of each antibiotic were mixed with each dilution of molasses in ratio 1:1 to determine the MIC of molasses in the presence of antibiotic then the MIC of molasses were mixed with each dilution of antibiotic (Cipo &Amp) in ratio1:1 to determined the MIC of antibiotic in the presence of molasses. Then by using agar well diffusion methods determined the antibacterial activity of MIC of molasses and antibiotic alone and in compensation through account of diameter of inhibition zone as design in [12].

2.4 Effect of pomegranate molasses on the bacterial virulence factors.
The bacterial suspension was mixed with MIC of molasses, and all tests for determine virulence factors were made as mentioned in step2.

2.5 Combination tests of molasses and antibiotic
The fractional inhibitory concentrations (FIC) was determined as described by Manda et al [14] to evaluate the effect of combination between pomegranate molasses and antibiotics on the antibacterial activity of each one, where the value calculated using the following formula:
FIC (antibiotic) = MIC of antibiotic in combination/MIC of antibiotic alone
FIC (extract) = MIC of extract in combination/MIC of extract alone
The interactions indices between the antibiotics and the molasses were assessed by using the FIC indices as described by Pankey et al.[15] and Kamatou et al.[16] which were calculated by using formula:
FIC index = ΣFIC = FIC (antibiotic) + FIC (molasses)
The combinations types were depended on value of FIC indices, so classified on this basis to several types such as synergistic (FIC indices were <1), additive (FIC indices were 1), indifferent (FIC indices were between 1 and 2), and antagonistic (FIC indices were >2).

3. Result and Discussion

3.1 Determine of the minimum inhibition concentration (MIC)
The minimum inhibitory concentration (MIC) of pomegranate molasses was 40 mg/ml, while was 70µg/ml for ciprofloxacin and 110 µg/ml for ampicillin (Table 1). The results show that molasses has
a good antibacterial effect compared with antibiotics. The MIC value of molasses decreased to 10 mg /ml when used with the ciprofloxacin antibiotic, while it was 20 mg /ml when used with the ampicillin antibiotic, indicating that the synergism use of the molasses with antibiotics has led to an increase effectiveness of molasses against pathogenic bacteria. The antibacterial activity of pomegranate molasses may be related to different compounds such as hydrolysable tannins, polyphenols and alkaloid, which was known as puricnine that play an important ability of the molasses to effect on bacteria by changing the protein nature that causes kills of bacteria [17]. The molasses contain several chemical compounds such as tannins( which display toxic effect on molecular level of the bacteria after cross the cellular wall of bacteria through their ability to precipitate proteins and inhibit the work of many enzymes such as glycosylate transferees) and polyphenol structures (which have directly influence on the bacterial cell wall and inhibit enzymes by oxidized agents in addition their ability to interact with proteins and disturb co-aggregation of microorganisms [18].

Table 1: MIC of pomegranate molasses and antibiotic against Serratia marcescens

| Type of material       | MIC of each material alone | MIC of molasses in combination with antibiotic | MIC of antibiotic in combination with molasses |
|------------------------|----------------------------|-----------------------------------------------|-----------------------------------------------|
| Pomegranate molasses   | 40 mg/ml                   | -                                             | -                                             |
| Ciprofloxacin          | 70µg/ml                    | 10 mg/ml                                      | 50 µg/ml                                      |
| Ampicillin             | 110µg/ml                   | 20 mg/ml                                      | 60 µg/ml                                      |
| D.W                    | 0                          | 0                                             | 0                                             |

3.2 Antibacterial activity of pomegranate molasses

The results showed clearly that pomegranate molasses were active against S. marcescens in comparison to antibiotic (ciprofloxacin and ampicillin) as a positive control and distilled water as a negative control (table 2). The rate diameter of inhibition zone of molasses reach to 1.5cm which regard good in compere to antibiotic that reach to 2.4 and 2cm for ciprofloxacin and ampicillin respectively. The results agree with Rathi namoorthy et al.[19] who attributed the antibacterial activity of pomegranate peel molasses to the presence of the broad spectrum antimicrobial compounds that act against both selected isolates. The result also showed that the mixture of antibiotic and molasses was more active against bacteria in comparison with antibiotic alone or molasses alone .The ability of plant to potentiate antibiotic has not been well describes. Where expected that it may be due to the inhibition of drug efflux and to many change in the bacterial cell wall that help the antibiotic in access to the target point [20].

Table 2. Antibacterial activity of Pomegranate molasses against Serratia marcescens bacteria

| Type of material       | Inhibition zone diameter rate (cm) |
|------------------------|-----------------------------------|
| Pomegranate molasses (g/ml) | 1.5cm                          |
| Ciprofloxacin MIC (µg/ml)     | 2.4 cm                          |
| Ampicillin MIC (µg/ml)        | 2 cm                            |
| MIC of Pomegranate molasses (gm/ml) and MIC of Ciprofloxacin (µg/ml) | 3.8 cm                          |
| MIC of Pomegranate molasses (gm/ml) and MIC of Ampicillin (µg/ml) | 3.1 cm                          |
| DW                      | 0                                |
3.3 Antivirulence activity of pomegranate molasses

The studied bacterial isolates had the ability to produce a number of virulence factors such as hemolysin, protease and biofilm. This ability was influenced by the addition of MIC of molasses or antibiotics to bacterial suspension. The results of the current study showed decline in the secretion of hemolysin toxin and protease enzyme after treatment compared to control. Figure 1 and 2 illustrates that *S. marcescens* was a high producer for biofilm formation; this was shown through its optical density that reaches to 1.8 when it was measured for bacterial suspension without molasses (regarded as control). However, the optical density showed reduced largely when the MIC of molasses (0.0588) or antibiotic such as ciprofloxacin (0.21) and ampicillin (0.016) was added to *S. marcescens* suspension before reading the optical density. While reached to 0.09 in compensation of molasses and ciprofloxacin and to 0.05 in compensation of molasses and ampicillin. The biofilm formation activity of *S. marcescens* bacteria were decline after treatment with MIC of molasses to transfer from high producer (OD high than 0.5) to producer (OD between 0.1-0.5) but when treated with molasses and MIC of ciprofloxacin antibiotic transfer to poor producer (OD lower than 0.1) in compare to bacteria treated with MIC of ciprofloxacin antibiotic only which transfer high producer to producer. Therefore, some plant extracts (such as pomegranate, which is one of the most important) are a good alternative to antibiotics in order to overcome the accelerating risk in the emergence of antibiotic-resistant pathogenic bacteria [21 & 5].

![Figure 1: Effect of molasses and ciprofloxacin on biofilm formation of bacteria.](image1)

![Figure 2: Effect of molasses and ampicillin on biofilm formation of bacteria.](image2)
3.4 Interactions between the molasses and antibiotics in vitro

The results of the current studies showed that the interaction between the studied antibiotics and molasses had positive effects in the elimination of pathogenic bacteria and found that the effectiveness of antimicrobial agents was improved by combining the antibiotics with pomegranate molasses against *S. marcescens* bacteria *in vitro*. As shown in Table 3, the interaction of molasses with ciprofloxacin was synergistic. Nevertheless, with ampicillin it was indifferent. In addition, showed that this compensation reduced MICs of antibiotics against resistant bacteria.

Some studies have indicated that there is a synergistic effect of most of the compounds extracted from medicinal plants when used with antibiotics. Some of them may be ineffective when used alone, while enhancing the activity of antibiotic when used against the pathogenic bacteria. In addition, the synergies between antibiotics and plant extracts may regard the first step to solve the toxicity problems of the human body resulting from the use of excess doses of antibiotics for the purpose of elimination of the high resistant pathogenic bacteria [22&23].

![Table 3. Effect of the combination between pomegranate molasses and antibiotics on *Serratia marcescens* bacteria.](image)

| Type of Antibiotic | Mean FIC (Antibiotic) | Mean FIC (Molasses) | FIC index | Interaction |
|-------------------|-----------------------|---------------------|-----------|-------------|
| Cip               | 0.714                 | 0.25                | 0.964     | Synergistic |
| Amp               | 0.545                 | 0.5                 | 1.045     | Indifferent |

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