Original Article:

The Antibacterial Properties of Aqueous and Ethanolic Extracts of *Punica granatum* Seeds on Bacterial Infectious Diarrhea

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ABSTRACT

Diarrhea is one of the gastrointestinal diseases which is associated with inappropriate antibiotic treatment and increased antibiotic resistance leading to problems in the health systems which varies in different societies. The present study aims to determine the antibacterial activity of aqueous and ethanolic extracts of pomegranate seeds (*Punica granatum*) on common gastrointestinal producing bacteria. Pomegranate seeds were harvested from Saveh city in a period of one year and were obtained between August 2015 and July 2016. After gathering, its aqueous and ethanolic extracts were prepared. The antibacterial property of aqueous and ethanolic extracts of *Punica granatum* seeds against 100 bacteria was considered. Minimum inhibitory concentration (MIC) of various concentrations (mg/ml) of the extracts was used as an index of antimicrobial activity. The MIC of aqueous extract vs. ethanolic was based on mg / ml were as follows: *E. coli* (75 vs 37.5), *Shigella sonnei* (37.5 vs., 18.75), *Shigella flexneri* (18.75 vs., 9.37), *Shigella dysentery* (18.75 vs., 9.37), *Proteus vulgaris* (18.75 vs., 9.37), *Proteus mirabilis* (9.37 vs., 9.37) and *Citrobacter fraundii* (150 vs., 75), respectively. The aqueous and ethanolic extracts had inhibitory effects on the studied bacteria, which depended on the concentration of the substances used. These inhibitory effects against each bacterium might depend on effective substances and the solvent properties. The present study suggests extracting new antimicrobial compounds by chromatography to obtain new antimicrobial agents.

Keywords: Antibacterial Properties; Aqueous Extracts; Ethanolic Extract; *Punica granatum* Seeds; Infectious Diarrhea; Bacteria

INTRODUCTION

Diarrheal disease is the second leading cause of death in children under five years of age, and is responsible for around 525000 deaths among children every year. Diarrhea can last several days, and the body losses a lot of water and salts that are necessary for survival. For most people, severe dehydration and fluid loss lead to the main causes of diarrhea deaths. Septic bacterial infections are likely to account for an increasing proportion of all diarrhea-associated deaths. Malnourished children or individuals with impaired immunity as well as people with HIV are most at risk of life-threatening diarrhea. Usually, three or more loose or liquid stools deification per day is defined as diarrhea. In breastfed babies, passing of neither formed stools nor "pasty" stools is not referred to as diarrhea. Diarrhea is caused by a variety of bacterial, viral and parasitic organisms. As a result of poor hygiene, infection is spread through improper sanitation, and hygiene, drinking of unsafe water, consumption of contaminated food; it can even be transferred from person to person directly. [1] Some common etiological agents of moderate-to-severe diarrhea in low-income countries are *Shigella sonnei*, *Shigella flexneri*, *Shigella dysentery*, *Proteus vulgaris*, *Proteus*

mirabilis, and Citrobacter fraundii. The purpose of this study was to investigate the antibacterial properties of *Punica granatum* seeds against bacteria causing gastrointestinal infections for the purpose of finding an alternative remedy.

The pomegranate, or *Punica granatum*, is a shrub that produces a red fruit. [2] Unique substances in pomegranate, such as punicalagins and punicic acid are responsible for most of health benefits. The juice and peels of a pomegranate contain punicalagins which are extremely powerful antioxidants. Pomegranate juice has been found to have three times antioxidant activity compared to red wine and green tea. Also, pomegranate seed oil, punicic acid, is the main fatty acid in the arils. It is a kind of conjugated linoleic acid with potent biological effects. [3]

Some chronic inflammations may be the cause for fatal diseases. These include heart disease, cancer, type 2 diabetes, Alzheimer's disease and even obesity. [4,6] One study based on diabetics patients found that 250 ml of pomegranate juice per day for 12 weeks lowered the inflammatory markers CRP and interleukin-6 by 32% and 30%, respectively .[7] Laboratory studies have shown that pomegranate extract can slow down cancer cell reproduction, and even induce apoptosis in cancer cells, especially prostate cancer. [8,9] Moreover, pomegranate extract causes inhibition of reproduction of breast cancer cells, and even destroys some of them. [10-12] Hypertension is one of main reasons for heart attacks and strokes. In one study, people with hypertension had a significant reduction after daily utilization of 150 ml (5 oz) of pomegranate juice for 2 weeks .[13] Other studies have proven the same effect, especially for systolic blood pressure.[14,15]

In western countries, arthritis is a common problem with many different types, where most of them are accompanied by some form of inflammation in the joints. Pomegranate anti-inflammatory effects help treat arthritis. Laboratory studies have proven that pomegranate extract can suppresses enzymes known for damaging joints in patients with osteoarthritis.[16,17] Pomegranate has also been identified as having beneficial effects against arthritis in mice; however, there has not been sufficient evidence in humans so far .[18,19] In addition, punicic acid of pomegranate may help protect against several steps in the heart disease process. In a study in 51 people with high cholesterol and triglycerides, 800 milligrams of pomegranate seed oil per day for 4 weeks significantly lowered triglycerides and improved the HDL ratio .[20] Another study on patients with type 2 diabetes and high cholesterol proved significant reductions in LDL cholesterol, as well as other improvements.[21]

Pomegranate juice has also been proved to protect the LDL cholesterol particles from oxidation, one of the key steps in the pathway towards heart disease.[22-25] Oxidative damage impairs blood flow in all areas of the body containing erectile tissue. In a study on rabbits, pomegranate juice has been shown to possess beneficial effects on increasing blood flow and erectile response. [26] In a study of men with erectile dysfunction, pomegranate had some benefit, but it was not statistically significant. [27] Some plant compounds in pomegranate can fight against harmful microorganisms. [28] The anti-bacterial and anti-fungal effects of pomegranate may also be protective against infections and inflammation of mouth such as gingivitis, periodontitis and denture stomatitis. [29, 30] There are some documents indicating that pomegranate can improve memory. In one study in surgical patients, 2 grams of pomegranate extract inhibited deficits in memory after the surgery .[31] Pomegranate is full of nitrates, which have been shown to improve exercise performance.[32] The purpose of this study was to investigate the antibacterial properties of aqueous and ethanolic extracts of *Punica granatum* seeds on bacterial infectious diarrhea**.**

MATERIALS AND METHODS

In this descriptive study, we included 100 outpatients who referred to Loghman Hakim Hospital with three or more loose or liquid stools deification per day in a period of one year during August 2015 and July 2016. Patients, who had received antibiotic treatment within the preceding 2 weeks were excluded. According to the following formula, a total of 100 bacteria isolated from outpatients with diarrhea during one year were evaluated:

$$n=\frac{Z^2_{1-\frac{\alpha}{2}}P(1-P)}{d^2}$$

53 *Escherichia coli*, 19 *Shigella sonnei*, 8 *Shigella flexneri*, 5 *Shigella dysentery*, 7 *Proteus vulgaris*, 5 *Proteus mirabilis* and 3 *Citrobacter fraundii* were isolated during one year. Stool samples were cultured on MacConkey agar and XLD agar. The predominant isolate on each plate (one colony) was selected, identified and stored in the -20 freezer for further analysis. [33, 34] Because this study was in line with patient treatment protocol and did not add any additional costs or pains to patients, no ethics committee approval was required.

Preparation of plant extracts

Pomegranates were gathered from Saveh city in a period of one year and obtained between August 2015 and July 2016. Then, its aqueous and ethanolic extracts were prepared.
The pomegranate seeds were collected after removing the skin, and placed in shadow, away from sunlight with appropriate temperature, until dried. [35]

A: Aqueous Extract

In order to prepare the aqueous extract, we first weighed 30 g of pomegranate seed powder and 100 ml of sterile distilled water in 70 to 80 ° C was added to the flask. Then, the foil was coated and placed inside water bath in 60° C. After 24 hours, the mixture inside the flask was compressed and straightened with a filter paper and a buckener funnel. [35, 36]

B: Ethanolic Extract

Extraction was carried out using percolation method. Fifty g of pomegranate seed powder was dispersed into separatory funnel, and then 70% ethanol was added stepwise. After 24 to 72 hours, it was time for seeds to be completely soaked and the maximum amount of its active ingredients was dissolved in ethanol for more extraction, and then the extract was isolated from the solvent through vacuum pump. [35, 36]

Determination of the minimum inhibitory concentration (MIC)

To determine the bacterial susceptibility to the pomegranate seed extract, MIC method was used. To prepare different dilutions of the extract, 300 mg of extract was first dissolved in 1 ml of normal saline, and then concentration of 300 mg /ml was obtained. Other dilutions such as 150, 75, 37.5, 18.75, 9.77, and 69.4 were prepared. To perform the MIC test, 80 µl of BHI broth, 100 µl of mentioned extract dilution, and 20 µl of bacterial suspension with 0.5 Mc Farland concentrations were added to each well. After 18 hours incubation, the lowest concentration which stops the growth of bacteria (lacking turbidity) was reported as the MIC. [36] MIC method was carried out in a 96-well ELISA plate. To ensure the precision of the results, test was carried out three times for each bacterial strain.

STATISTICS

As the study was a descriptive one, there was no need to state about statistical methods and software.

RESULTS

A total of 100 bacteria were studied as following: 53 *E. coli*, 19 *Shigella sonnei*, 8 *Shigella flexneri*, 5 *Shigella dysenteriae*, 7 *Proteus vulgaris*, 5 *Proteus mirabilis*, and 3 *Citrobacter fraundii*. The minimum inhibitory concentrations of aqueous / ethanolic extracts (mg / ml) for *E. coli* (75 vs., 37.5), *Shigella sonnei* (37.5 vs., 18.75), *Shigella flexneri* (18.75 vs., 9.37), *Shigella dysenteriae* (18.75 vs., 9.37), *Proteus vulgaris* (18.75 vs., 9.37), *Proteus mirabilis* (9.37 vs., 9.37), and *Citrobacter fraundii* (150 vs., 75) were shown in tables 1and 2.

Table1. Evaluation of antimicrobial susceptibility of aqueous extract on studied strains

Species / Extract Concentration	300 mg/ml	150 mg/ml	75 mg/ml	37.5 mg/ml	18.75 mg/ml	9.37 mg/ml	4.69 mg/ml
E. coli	+[1]	+	+	-[2]	-	-	-
Shigella sonnei	+	+	+	+	-	-	-
Shigella flexneri	+	+	+	+	+	-	-
Shigella dysentery	+	+	+	+	+	-	-
Proteus vulgaris	+	+	+	+	+	-	-
Proteus mirabilis	+	+	+	+	+	+	-
Citrobacter fraundii	+	+	-	-	-	-	-

+1: Susceptible
-2: Resistant

Table 2. Evaluation of antimicrobial susceptibility of ethanolic extract to studied strains

Species / Extract Concentration	300 mg/ml	150 mg/ml	75 mg/ml	37.5 mg/ml	18.75 mg/ml	9.37 mg/ml	4.69 mg/ml
E. coli	+[1]	+	+	+	-[2]	-	-
Shigella sonnei	+	+	+	+	+	-	-
Shigella flexneri	+	+	+	+	+	+	-
Shigella dysentery	+	+	+	+	+	+	-
Proteus vulgaris	+	+	+	+	+	+	-
Proteus mirabilis	+	+	+	+	+	+	-
Citrobacter fraundii	+	+	+	-	-	-	-

+1: Susceptible
-2: Resistant

DISCUSSION

According to our study, the aqueous and ethanolic extracts have inhibitory effects on the studied bacteria, which depends on substances concentration. Increasing occurrence of drug resistance between bacteria, distribution of resistance encoding genes among nosocomial infection producing strains, and several adverse effects of synthetic and chemical drugs can cause a requirement for identification and correct utilization of herbal remedies with antimicrobial effect to cope with infections or to control the side effects of synthetic drugs. [37]

Similar to our study on the antibacterial properties of pomegranate seeds, Al Laham SA and Al Fadel in 2013 studied the anti-bacterial effect of *Punica granatum* extracts against antibiotic resistant *Pasteurella haemolytica*. The ethanolic extracts prepared from different parts of *P. granatum* showed the high antibacterial effectiveness, where the pericarp extract was the best, and the water and ether petroleum extracts had no antibacterial effect against resistant *P. haemolytica*. Ethanol extracts of *P. granatum* (pericarp, leaves, flowers, seeds) had antibacterial effects against *P. haemolytica* which showed resistance to all studied antibiotics.[38]

Also, the other study was conducted by Growther L and et al., which also took place on *E.coli*, suggested phytochemicals can be used as an alternative to antibiotics. According to their study, *Punica granatum* peels were rich in polyphenols, tannins and other secondary metabolites. They analyzed the bioactive phyto-components of *Punica granatum* peel extracts against Shiga -toxin producing *E. coli*. The methanol extract of *P. granatum* peel showed high antibacterial activity against STEC. [39]

Nuamsetti T and et.al in 2012 investigated in vitro antibacterial activities of different extracts of pomegranate fruit peels and arils (with seeds) investigated by agar-well diffusion and broth dilution methods against four food-related bacteria (*Bacillus subtilis*,*Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*). The solvents used as extractants were hot water, 95% ethanol, and acetone. All pomegranates extracts contained high levels of phenolic compounds and exhibited antibacterial activity against all tested bacteria. The hot-water extract of the peels was the most potent with the MIC of 207 mg/ml against *E. coli* and less than 103.6 mg/ml against the other bacteria. Gram-positive bacteria were generally more sensitive to the extracts than Gram-negative ones. [40] In the present study, *E.coli* was inhibited with a higher concentration of extract, which could be due to bacterial isolation source, drug resistance of bacteria and type of extraction method.

Based on Elaleem study in 2016, the various fruit rind extracts of *Punica granatum* revealed triterpenoids, steroids, flavonoids, fannins, faponins, alkaloids and glycosides. Methanol extract of pomegranate fruit rind showed high antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* , *Escherichia coli* and *Proteus vulgaris* respectively, compared to chlorophorm and water extract. [41] Similar to our study, the alcoholic extract had an antibacterial effect more than the aqueous extract.

The antimicrobial properties of pomegranate have been proven by different methods and distinct studied population. Abdollahzadeh et.al evaluated antibacterial and antifungal activities of *Punica granatum* peel extracts against oral pathogens. The antimicrobial activity was measured by agar disk diffusion method. All concentrations of *Punica granatum* had antibacterial activity against *S. aureus* and *S. epidermidis*, but only some concentrations of *Punica granatum* were effective against *Lactobacillus acidophilus*, *Streptococcus mutans* and *Streptococcus salivarius*. Furthermore, no concentrations of *Punica granatum* inhibited *A. viscosus* and *Candida albicans*. They suggested that *Punica granatum* might be used as an antibacterial agent in controlling oral infections. [42]

Sharma HK et.al in 2014 found that pomegranate played a very important role in Indian fruit market. Furthermore, they used *Punica granatum* seeds, pericarp (peel) as well as juice to evaluate the antibacterial activity against enteric bacteria i.e. *E. coli*. For the detection of antibacterial activity, agar gel diffusion method was used. As a result, zone of inhibition was only shown by seed. On the other hand, zone of inhibition was not observed in juice and peel. So they concluded that the granatum seeds persists the anti-bacterial property. Secondly, solvent extract of the pomegranate peel i.e. water, ethanol and methanol extracts were prepared and antibacterial activity was tested against enteric bacteria *E. coli*. As a result, methanol and ethanol extract showed almost same zone of inhibition while water extract showed comparatively less zone of inhibition. [43] Similar to our research results, the anti-bacterial property of granatum seeds was stable and the antibacterial activity of alcoholic extracts were comparable significantly to water extract against tested bacteria.

The quality of the natural extracts and their antioxidant effects depends not only on storage and maintenance, the geographical origin and the time of treatment, but also on the environment technological factors used in extraction, and even the type of solvent used. In the other hand, the antioxidant effects of plant extracts play important role because different antioxidant agents have different polarities. Furthermore, several types of resistant bacteria may show different results with a distinct concentration of extract [44-47]

As mentioned above, there are various reasons why antimicrobial effect of this plant varies; they include the method of extraction and even solvent properties, plant species, geographical location, stresses introduced into the plant, intrinsic differences, and the amount and type of chemical compounds of the plant.

CONCLUSION

The process of bacterial resistance to conventional chemical antibiotics has limited the ability of physicians to treat some infectious diseases that are often threatening. Through focusing on the results, medicinal plants should be used in accordance with the advancement of science and technology. Since pomegranate has varied medical effects, it should be considered by researchers in different parts of medical sciences. It is suggested to extract new antimicrobial compounds through chromatography.

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