

## Full Length Research Paper

# Antimicrobial activity of pomegranate rind peel extracts

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The pomegranate, *Punica granatum* L., is an ancient, mystical, unique fruit borne on a small, long-living tree cultivated throughout the Mediterranean region. Pomegranate is used in several systems of medicine for a variety of ailments. The synergistic action of the pomegranate constituents appears to be superior to that of single constituents. *P. garantum*, have been reported to have antimicrobial activity against a range of Gram positive and negative bacteria. Pomegranate formulations containing ferrous salts have enhanced although on short-term. The aim of this experiment is to determine the antimicrobial activities of combinations of pomegranate rind extract with range of metal salts with the addition of vitamin C. Phytochemical analyses was made to determine the active inhibitors in rind extract, including phenolics and flavonoids.

**Key words:** Antimicrobial activity, pomegranate rind extract, phenolics, flavonoids.

## INTRODUCTION

*Punica granatum* L. (*Punicaceae*), commonly called pomegranate, recently described as nature's power fruit, is a plant used in folkloric medicine for the treatment of various diseases (Abdel Moneim et al., 2011; Ajaikumar et al., 2005) widely cultivated in the Mediterranean region. Pomegranate has strong antioxidant and anti-inflammatory properties, recent studies have demonstrated its anti-cancer activity in several human cancers (Adhami and Mukhtar, 2007; Longtin, 2003). In addition, pomegranate peel extract with an abundance of flavonoids and tannins has been shown to have a high antioxidant activity (Abdel Moneim et al., 2011).

Antimicrobial drug resistance in human bacterial pathogens is a worldwide issue and as a consequence, effective treatment and control of such organisms remain an important challenge. Bacterial resistance has appeared for every major class of antibiotic (Lambert, 2005). Since their introduction the emergence of resistant is evident, particularly for important pathogens such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Enterococcus* spp. and *Staphylococcus* spp. (Musgrove

et al., 2006).

Over the last decade research into the antimicrobial properties of traditional plant based medicines has been revisited (Melendez and Capriles, 2006; Navarro et al., 1996). Numerous plants have been screened for antimicrobial properties, for example Holetz et al. (2002) tested 13 plants used in Brazilian traditional medicine and they demonstrated activity against bacteria such as *Staphylococcus aureus* and *E. coli*. Melendez and Capriles (2006) tested 172 plant species used in Puerto Rico and they demonstrated that 14 of these showed activity against bacteria including *S. aureus* and *E. coli*. Prashanth et al. (2001) tested a number of extracts of pomegranates against a range of bacteria (*S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Bacillus subtilis* and *Salmonella typhi*), and they found activity against all isolates. Braga et al. (2005) observed that pomegranate extracts were able to inhibit not only the growth of *S. aureus* but also the production of enterotoxin. The methanolic extract derived from 200 g of dried pomegranate produced bactericidal effects at 1% (v/v) over an extended incubation period (50 h), demonstrating longevity of action.

Many bacteria have advanced protective mechanisms for the detoxification of heavy metal ions (Silver, 1996). Despite this, numerous literature reports address the

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development of metal compounds as antimicrobial agents. Many low molecular-mass metal compounds exhibit bactericidal and/or bacteriostatic activities. In one study the susceptibilities of *Staphylococcus* strains to solutions of metal salts (in the range of 50  $\mu\text{mol}$  to 80  $\text{mmol}$ ) were determined and frequencies of resistance were found to be  $\text{CuSO}_4$  and  $\text{NiCl}_2$ , 36.2%;  $\text{ZnSO}_4$ , 13.6% and  $\text{CoCl}_2$ , 4.5%, respectively (Ug and Ceylan, 2003).

## MATERIALS AND METHODS

### Preparation of pomegranate rind extracts

Pomegranate rind extracts (PRE) were prepared by blending 50 g of the seed with 100 mls of distilled water for 10 min. The crude extract was filtered through muslin followed by Whatman No. 1 filter paper prior to autoclaving (121°C for 15 mins) before storage at -20°C (Stewart et al., 1998).

### Test for tannins

The aqueous extract (1 ml) was mixed with 10 ml of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins or catechol tannins, respectively.

### Reducing power

The  $\text{Fe}^{3+}$  reducing power of the extract was determined according to the method of Oyaizu (1986). The extract (2 ml) was mixed with 0.2 M phosphate buffer, pH 6.6 (2 ml) and 1% potassium ferricyanide (2 ml). The mixture was then incubated at 50°C for 20 min. Afterwards, the mixture was stopped by adding 10% trichloroacetic acid (2 ml) and then centrifuged at 3,000 rpm for 10 min. The upper layer of supernatant (2 ml) was mixed with distilled water (2 ml) and 0.1%  $\text{FeCl}_3$  solution (0.5 ml). The absorbance was measured at 700 nm against a blank with a spectrophotometer, and ascorbic acid was used as a standard. Higher absorbance of the reaction mixture indicated greater reducing power. The percents of reducing power were presented as ascorbic acid equivalents using a calibration curve between the absorbance of the reaction and the percent of the reducing power ability of ascorbic acid:

$$\text{OD} = (0.0146 \times [\text{percent}]) + 0.0016, R^2 = 0.9999$$

### Determination of total flavonoids

For the assessment of flavonoids, colorimetric method introduced by Dewanto et al. (2002) was adapted. To determine the amount of flavonoids by the above mentioned method, 1.50 ml of the deionized water was added to 0.25 ml of the sample extract and then 90  $\mu\text{l}$  of 5% Sodium nitrite ( $\text{NaNO}_2$ ). Six minute later, after addition of 180  $\mu\text{l}$  of 10%  $\text{AlCl}_3$ , mixture was allowed to stand for another 5 min before mixing 0.6 ml of 1 M NaOH. By adding deionized water and mixing well, final volume was made upto 3 ml. Using blank, absorbance was measured at 510 nm. Calibration curve was prepared using quercetin acid as standard for total flavonoids which was measured as milligram quercetin equivalents (QE) per gram of the sample (mg/g).

### Determination of total phenolics

To analyze the total phenolic content (TPC), Kim et al. (2003)

method was followed to make the use of Folin Ciocalteu reagent. To 0.4 ml of the extract (prepared in methanol with a concentration of 1.0 mg/ml), 1.0 ml of (10%) Folin-Ciocalteu reagent was mixed and solution was allowed to stand at 25°C for 5 to 8 min before adding 0.8 ml of 7.5% sodium carbonate solution and using deionized water, final volume was made to 10.0 ml. After two hours, absorbance was measured at 765 nm. Calibration curve was prepared using gallic acid as standard for TPC which was measured as mg gallic acid equivalents (GAE) per gram of the sample (mg/g).

### Antimicrobial activity of PRE/metal salt combinations

A sample of the PRE was preserved in refrigerator at 4°C until used. An aliquot of PRE (330  $\mu\text{l}$ ) was added to (700  $\mu\text{l}$ ) solutions (4.8 mM) of each metal salts used ( $\text{CuSO}_4$ ,  $\text{MnSO}_4$  and  $\text{ZnSO}_4$ ) and the final solution was protected from light (Stewart et al., 1998).

### Antimicrobial activity of PRE/metal salt combinations plus vitamin C

The assay was carried out as described above with the following addition: A sample of the PRE was added to vitamin C (1:1), sample of the PRE (330  $\mu\text{l}$ ) was added to (330  $\mu\text{l}$ ) of vitamin C and to the metal salt 330  $\mu\text{l}$  of ( $\text{CuSO}_4$ ,  $\text{MnSO}_4$  and  $\text{ZnSO}_4$ ) solution immediately prior to mixing.

### Screening assay

Overnight cultures of the Gram positive strains *Staphylococcus* spp., *B. subtilis*, *Bacillus indicus* and the Gram negative strains *E. coli*, *Enterobacter aerogenes*, *Serratia marcescens* and *Brucella* spp. were prepared on nutrient agar plates (Oxoid Ltd, UK). All bacterial isolates were suspended in Ringer's solution (Oxoid Ltd, UK) to a turbidity equivalent to 0.5 McFarland (1.5  $\times$  10<sup>8</sup> CFU/ml) and 100  $\mu\text{l}$  were spread onto Mueller-Hinton agar plates (Oxoid Ltd, UK). Also yeasts as *Saccharomyces cerevisiae* and *Rhodotorula glutinis* and *Geotrichum* spp. The PRE (10  $\mu\text{l}$ ) was then spotted onto sterile Whatman no 1 filter paper discs (5 mm diameter) placed centrally on the plates which were incubated at 37°C for 24 h prior to recording zones of inhibition (McCarrell et al., 2008).

## RESULTS AND DISCUSSION

Pomegranate is an important source of anthocyanins, hydrolysable tannins punicalagin and punicalin (Afaq et al., 2005), ellagic and gallic acids (Lansky and Newman, 2007) and also contains vitamin C (Turk et al., 2008). The antioxidant and free radical scavenging activity of phenolic compounds derived from pomegranates (Rosenblat et al., 2006) and vitamin C (Sonmez et al., 2005) have been reported (Abdel Moneim, 2011). The PRE gave positive tests for gallic tannins while the extract gave negative result for catechol tannins as shown in Table 1. The reducing power (RP) and the total phenolic (TPC) and flavonoids (TFC) contents in pomegranate rind extracts are shown in Table 2. The direct correlation between antioxidant activity and reducing power of certain plant extracts were reported. The presence of reductants (antioxidants) in the extracts

**Table 1.** Quantitative analysis of tannins and its type in the rind extract of pomegranate.

Parameter	Amount
Gallic tannins	++
Catechol tannins	-

**Table 2.** Reducing power (%/ g sample), total phenolic (mg GAE/g sample) and flavonoids (mg QE/ g sample) contents in the rind extract of pomegranate.

Parameter	Content
Reducing power (RP)	14.65±1.34
Total phenolic content (TPC)	104.68±3.21
Total flavonoids contents (TFC)	47.27±1.54

<sup>a</sup> Values expressed are the mean of three replications.

would result in the reduction of iron (III) to iron (II). PRE showed 14.65% of activity at 1 g sample/ml. The result indicates that PRE contained electron donors and possessed the ability to reduce iron (III) to iron (II).

It was well-known that plant phenolics and flavonoids are highly effective free radical scavengers and antioxidants. *P. granatum* contained high amounts of phenolic and flavonoids compounds. It was shown that PRE contained phenolic and flavonoids compounds at 104.68 mg GAE/g and 47.27 mg QE/g, respectively (Table 2). This result indicates that the potent antioxidant activity of PRE may be related to the phenolic and flavonoids compounds in the extract.

It has been reported that oxidizing biological material leads to a rapid burst of ROS, such as superoxide ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl ( $\cdot OH$ ) generated primarily because of the ionizing of water molecules (Agrawal et al., 2001), which then interact with biological target molecules, causing lipid peroxidation and DNA damage, and subsequently resulting in cell killing and mutations (Abdel Moneim et al., 2011).

Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted currently as one of the main sources of chemo preventive drug discovery and development (Aruoma, 2003). It has been observed that many plant polyphenols, such as ellagic acid, catechins, and chlorogenic, caffeic and ferulic acids act as potent antioxidant, antimutagenic and anticarcinogenic agents (Ayrton et al., 1992).

Data in Table 3 indicates antimicrobial activity of the different mixture of PRE, vitamin C and metals after autoclaving as separated and mixed components where the PRE/ vitamin C (1/1), exhibited antimicrobial activity against Gram positive (*B. indicus*) and Gram negative (*E. coli*) organisms reached 18 and 20 mm respectively. While mixing PRE/ $ZnSO_4$  showed antimicrobial activity

against *B. subtilis*, *Staphylococcus* spp. and *Brucella* spp. and were 15, 15 and 15 mm respectively. PRE/ $MnSO_4$  affected only on the yeast *Saccharomyces cerevisiae*. Moderate antimicrobial activity was seen with the mixing of PRE/vitamin C /salts metals on the different micro-organisms. Results indicated that PRE/vitamin C/  $MnSO_4$ , just effected on *S. cerevisiae* and the zone of inhibition was 10 mm whilst PRE/vitamin C/  $CuSO_4$  effected on *Brucella* spp. and gave 10 mm zone of inhibition. PRE/vitamin C/  $ZnSO_4$  were not effected on any of the tested strains. The PRE alone did not exhibit anti-microbial activity against any of the isolates. Variations in results between studies on pomegranate extracts are not only seen in disc diffusion assays, but have also been recorded with minimum inhibition concentration (MIC) determinations.

No detectable effect of any of the extraction mixture on the growth of *Enterobacter aerogenes* and the yeast *Rhodotorula glutinis*. McCarrell et al. (2008) prepared ethanol extractions of a number of plants and tested these against a range of laboratory and clinical isolates. Interestingly, this group only reported antimicrobial activity for pomegranate extracts against laboratory strains of *P. aeruginosa* and *B. subtilis*. In their test, *B. subtilis* produced a zone of clearing equal to or greater than 7 mm. These differences may in part be due to the different extraction methods employed, potentially the freshness of the fruit used, and variations in the season and region of growth.

Melendez and Capriles (2006) tested the antimicrobial properties of a number of tropical plants from Puerto Rico using the disc diffusion method against *E. coli* and *S. aureus*. They demonstrated that pomegranate extract produced inhibition zone sizes of 11 and 20 mm, for *E. coli* and *S. aureus* respectively. Thus, their results contrast to the present study in that a smaller zone of inhibition for *S. aureus* was observed along with antimicrobial activity against *E. coli*. In the present work the value of MIC was 0.5 mg/l against all positive results that was mentioned in Table 2. Values for MIC have been reported in a number of studies, ranging from 0.62 to 10 mg/ml against *S. aureus*, *E. coli* and *P. aeruginosa* (Navarro et al., 1996) and up to 250 mg/L against *S. aureus* (Machado et al., 2003). These differences could be due to the extraction method, freshness of the fruit, season and region of growth (McCarrell et al., 2008).

## Conclusion

Combinations of PRE with metal salt  $ZnSO_4$  exhibit enhanced antimicrobial effects against *Bacillus subtilis*, *Staphylococcus* spp. and *Brucella* spp. Mixture of PRE/ Vitamin C (1:1) exhibited higher zone of inhibition against *E. coli* and *B. indicus* in comparison to the three mixtures. Further work is underway to establish the mode of action, along with the mechanism of enhancement by metal salts

**Table 3.** Diameter of the zones of inhibition (mm) of the autoclaved pomegranate rind extract compared to penicillin and the results.

Tested organisms	PRE/ Vitamin C (1:1)	PRE/ MnSO <sub>4</sub> (330/700 µl)	PRE/ CuSO <sub>4</sub> (330/700 µl)	PRE/ ZnSO <sub>4</sub> (330/700 µl)	PRE/ Vitamin C/ MnSO <sub>4</sub> (1/1/1)	PRE/ Vitamin C/ CuSO <sub>4</sub> (1/1/1)	PRE/ Vitamin C/ ZnSO <sub>4</sub> (1/1/1)
<i>E. coli</i>	20	0	0	0	0	0	0
<i>Bacillus subtilis</i>	0	0	0	15	0	0	0
<i>Bacillus indicus</i>	18	0	0	0	0	0	0
<i>Enterobacter aerogens</i>	0	0	0	0	0	0	0
<i>Serratia marcescens</i>	0	0	0	0	0	0	0
<i>Brucella spp.</i>	0	0	7	15	0	10	0
<i>Staphylococcus spp.</i>	0	0	0	15	0	0	0
<i>Saccharomyces cerevisiae</i>	0	13	0	0	10	0	0
<i>Rhodotorula glutinis</i>	0	0	0	0	0	0	0

O: No effect on the growth of the microorganisms.

and vitamin C.

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