

Antityphoid Activity of Peel, Pericarp and Fruit Extracts of *Punica Granatum* (Pomegranate) in vitro

MARYAM RASHID, AMBREEN SHABBIR, RABIEA BILAL, AMNA HAFEEZ, ABDUL HANNAN*.

ABSTRACT

Aim: To evaluate the anti-bacterial potential of pomegranate against *Salmonella typhi*.

Methodology: Ethanolic extracts of pomegranate's different parts; peel, pericarp and fruit were screened for anti-bacterial activity against Multi-Drug Resistance (MDR) strain (UHS-14) by agar well diffusion method. 6% phenol was used as positive control.

Results: The peel extract had the largest inhibitory zone of 22.73±0.26mm against *Salmonella typhi*, followed by pericarp (22.47±0.36mm) and fruit (15.55±0.29mm) extracts at neat concentration. The peel, pericarp and fruit extracts were further evaluated for minimum inhibitory concentration (MIC) against forty five clinical isolates of *Salmonella typhi*. The peel extract showed MIC of 22mg/ml; followed by pericarp (MIC 24mg/ml) and fruit extract (64mg/ml).

Conclusion: Ethanolic peel extract displayed highest antibacterial activity against *Salmonella typhi* in both agar diffusion and agar dilution assay as compared to pericarp and fruit extracts. This warrants further evaluation of peel extract in suitable typhoid animal model.

Keywords: Pomegranate, typhoid fever, multidrug resistance (MDR).

INTRODUCTION

Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype Typhi (*S. typhi*). Typhoid fever is endemic in Pakistan and remains a common cause of hospital admission¹. In 1948, chloramphenicol was introduced as the first effective antibiotic in the treatment of typhoid fever but resistance started to develop within two years of its introduction, though not as a major problem until 1972². Amoxicillin and co-trimoxazole were introduced as the effective alternative anti-typhoid till the development of "multidrug resistant" (MDR) strains (resistant to ampicillin, chloramphenicol and cotrimoxazole) towards the end of 1980s and early 1990s. High rates of "MDR" strains were recorded as 70% from Pakistan³. The worsening situation was saved by the introduction of the fluoroquinolones¹³. Fluoroquinolones have been considered the treatment of choice for the enteric fever. Ofloxacin was reported to be extremely effective against typhoid fever in 1986 and fluoroquinolones continued to be useful antibiotics⁴. Alarming reduced sensitivity to the fluoroquinolones which have been widely used to treat such "MDR" strains, has appeared and continues to spread⁵.

Researchers now are increasingly turning their attention to folk medicine looking for new leads to develop better drugs against cancer, as well as viral and microbial infections. Although thousands of plant

species have been tested for antimicrobial properties, the vast majority have not yet been adequately evaluated.⁶ *Punica granatum* L, known as pomegranate belongs to the family Puniceae. The pomegranate plant possesses an immense therapeutic value. A number of biological activities such as antitumour, antibacterial, antidiarrhoeal, antifungal, antiulcer have been reported with various constituents/extracts of different parts of this plant⁷.

Pomegranate fruit extract is a rich source of 2 types of polyphenolic compounds: anthocyanins (such as delphinidin, pelargonidin and cyanidin), which give the fruit and juice its red color, and the hydrolysable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose), which account for 92% of the antioxidant activity of the whole fruit⁸. Extracts from many parts of the plant such as juices, peel and seed oil have been reported to exhibit strong anti-oxidant activities attributed to its high content of polyphenolics including ellagitannins and ellagic acid.⁹ Studies have shown that *Punica granatum* is effective at inhibiting gram-positive as well as gram-negative bacterial growth. Anti-bacterial activity of petroleum ether, methanol, chloroform and water extracts of pomegranate peel was tested. However, the methanolic extract was found to be the most effective against all tested microorganisms such as *Staphylococcus aureus*, *E.coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhi* and *Bacillus subtilis*¹⁰ And the reason for this anti-

*Department of Microbiology & Pharmacology, University of Health Sciences, Lahore.

Correspondence to Major General (retd) Prof. Abdul Hannan, Head of Department of Microbiology,

bacterial activity of all the pure compounds was attributed to their phenolic structure¹¹.

The different parts of pomegranate have been tested for their antimicrobial action against various microorganisms, yet no such research has been done regarding the comparison of its different parts for their anti-bacterial activity against different species of *Salmonella typhi* including "MDR" *Salmonella typhi* which is of great concern in Pakistan.

MATERIAL & METHODS

Forty five clinical isolates of *Salmonella typhi* were used. These were obtained from the Department of Microbiology, Armed force institute of Pathology (AFIP), Rawalpindi and Sheikh Zayed Hospital, Lahore. 6% phenol and propylene glycol were used as positive and negative controls respectively. Susceptibility was done by Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines using commercially available antimicrobial disks. Five kilograms of fresh *Punica granatum* (Kandhari anar) were thoroughly washed before cutting and separating into its portions; peel, pericarp and fruit. The washed components were air dried for 2 weeks. Peel, pericarp and fruit were crushed in mechanical mortar and weighed. Peel, pericarp and fruit weighed 770 grams, 40 grams and 830 grams respectively. These were dipped in ethanol for 7 days in separate containers. Peel, pericarp and fruit were dipped in 1500 ml, 300 ml and 450 ml ethanol respectively. After a week, these were filtered through filter paper. The solvent was then removed under reduced pressure in a rotary evaporator. In the rotary evaporator; the extracts were then passed through a water bath at 40° C and chiller at 8° C at the speed of 85 rpm/ second until the solvent was evaporated. Three extracts were weighed again and were kept in 3 separate containers. The percentage yield was determined; 20% for peel, 36% for pericarp and 30% for fruit^{12,13}. The extracts were prepared from Pakistan council of scientific and industrial research (PCSIR) laboratories, Lahore.

One *Salmonella typhi* strain (UHS 14) was selected for screening the inhibitory effects of the pomegranate extracts on MDR *S. typhi*. McFarland 0.5 standard was used for inoculum preparation. After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjacent suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The dried surface of a Mueller-Hinton agar plate was streaked. Four wells of 9mm in diameter were made with sterile cork borer

on each previously seeded MH agar plate. Each well was filled with 120µl of pomegranate extracts of different concentrations (2%, 4%, 8%, 16%, and 32%) which were made in propylene glycol. An equal quantity of diluent was filled in wells as negative control. The plates were incubated at 35°C and zone of inhibition was measured after 18 hours with a digital caliper. The study was performed in triplicate and mean value was calculated¹⁴.

The agar dilution assay method was used to determine the MIC of the peel, pericarp and fruit extracts. The extracts were tested against 45 different strains of *Salmonella typhi*. Mixing of pomegranate extracts with autoclaved MH agar were performed at 50°C, vigorously vortexed and dispensed into Petri dishes. The poured plates were allowed to dry at 45°C for about 10 to 15 minutes. Four to five well separated colonies from overnight blood agar were emulsified in 5 ml of sterile distilled water, adjusting to 0.5 McFarland's standard. The extract incorporated plates were inoculated with multipoint inoculator. The plates were incubated at 37°C for 18 hrs and observed for growth¹⁵.

The MIC was recorded as the lowest concentration of pomegranate extract at which visible bacterial growth was completely inhibited. This experiment was performed in triplicate to ensure the reproducibility of the results.

The data was entered and analyzed using SPSS 16.0. Mean ± S.D is given for quantitative variables. Frequencies, percentages and graphs are given for qualitative variables. One way analysis of variance (ANOVA) was applied to observe mean differences. Post HOC Tukey test was applied to observe which group means differs. Two independent sample T test was applied to observe group mean differences between two groups. A *p*-value of <0.05 was considered to be statistically significant.

RESULTS

The antibacterial effect of pomegranate extracts; peel, pericarp and fruit on MDR *Salmonella typhi* (UHS14) is shown in table 3. The mean zone of inhibition of peel extract was 22.73±0.26 at neat (100%) concentration and it was 22.47±0.36mm and 15.55±0.29mm for pericarp and fruit extract respectively; the peel extract showing the greatest inhibitory zone. According to table 4, 6% phenol produced a zone of inhibition of 16.62±0.26mm and propylene glycol showed no zone of inhibition.

Table 3 shows that at 16mg/ml of the peel extract only one strain of *Salmonella typhi* was inhibited. At 20mg/ml; two strains were inhibited and at 22mg/ml all the *Salmonella typhi* were inhibited. So the MIC range of the peel extract was 16-22mg/ml.

Table 4 shows that at 14 and 16mg/ml of the pericarp extract one strain of *Salmonella typhi* was inhibited. At 18mg/ml of the pericarp extract; five strains of *Salmonella typhi* were inhibited. At 22mg/ml; one strain was inhibited and at 24mg/ml; thirty seven strains of *Salmonella typhi* were inhibited. So the MIC range of the pericarp extract was 14-24mg/ml.

Table 5 shows that at 54mg/ml of the fruit extract five strains of *Salmonella typhi* were inhibited. At 64mg/ml; forty strains were inhibited. So the MIC range of the fruit extract was 54-64mg/ml.

The values represent mean \pm standard deviation of three replicates, * Not inhibited

Graph 1: Correlation between zone size (mm) and concentration of the extracts (%) against MDR *Salmonella typhi* (UHS 14) by agar well diffusion method

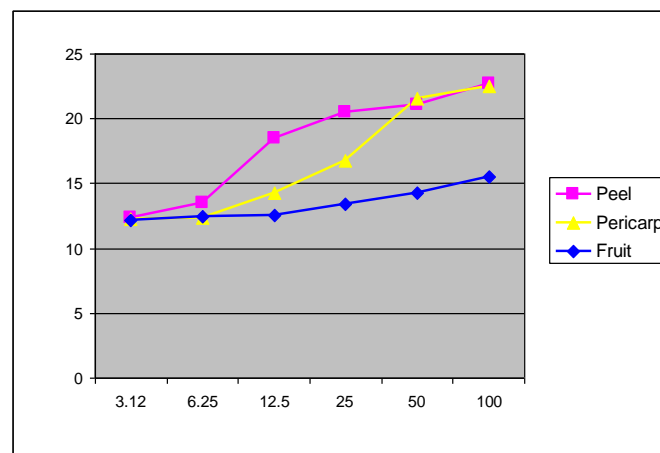


Table 1: Measurement of zone of inhibition of peel, pericarp and fruit extracts against MDR *Salmonella typhi* (UHS 14) by agar well diffusion method.

Dilutions of extracts (%)	Peel	Pericarp	Fruit
100	22.73 \pm 0.26*	22.47 \pm 0.36	15.55 \pm 0.29
50	21.03 \pm 0.46	21.57 \pm 0.38	14.24 \pm 0.38
25	20.54 \pm 0.29	16.72 \pm 0.23	13.38 \pm 0.07
12.5	18.49 \pm 0.30	14.24 \pm 0.39	12.56 \pm 0.49
6.25	13.51 \pm 0.13	12.37 \pm 0.24	12.48 \pm 0.46
3.12*	12.38 \pm 0.53	12.26 \pm 0.70	12.21 \pm 0.38

P value: <0.001, *0.924

Table 2: Measurement of zone of inhibition of controls by agar well diffusion method against multidrug resistant *Salmonella typhi* (UHS 14)

Controls	Zone of inhibition (mm)
Phenol 6%	16.62 \pm 0.26
Propylene glycol	NI*

Table 3: Number of isolates inhibited at different concentrations (mg/ml) of peel extract.

Isolate type & No.	1.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0
<i>S. typhi</i> (n=45)	0	0	0	0	0	0	0	0	1	0	2	42

MIC range: 16-22, MIC₅₀: >20, MIC₉₀: 22

Table 4: Number of isolates inhibited at different concentration (mg/ml) of pericarp extract.

Isolate type & No.	1.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0
<i>S. typhi</i> (n=45)	0	0	0	0	0	0	0	1	1	5	0	1	37

MIC range: 14-24, MIC₅₀:>22, MIC₉₀:24

Table 5: Number of isolates inhibited at different concentration (mg/ml) of fruit extract.

Isolate type and No.	10	12	14	16	18	20	22	24	26	34	44	54	64
<i>S. typhi</i> (n=45)	0	0	0	0	0	0	0	0	0	0	0	5	40

MIC range: 54-64, MIC 50: >54, MIC90: 64

DISCUSSION

Pomegranates have been used extensively in ancient cultures for various medicinal properties. Regarding the Qualitative analysis of the extracts; peel, pericarp and fruit was done by agar well diffusion method. One "MDR" strain of *S. typhi* (UHS 14) was tested for its susceptibility to the three extracts. It was seen that

increasing the concentration of the extract, increased the zone size as seen in table 3 of the results. The peel extract showed the inhibitory zone of 12.38 \pm 0.53mm at the lowest concentration of the extracts (3.12%) which was greater than the zone size produced by fruit and pericarp extracts. Thus it was deduced from the results of agar well diffusion

method that peel extract had the greatest inhibitory activity against "MDR" *S. typhi*.

S. Naz *et al* identified and tested a number of active ingredients from methanolic extract of pomegranate. These pure compounds were tested against a number of gram positive and gram negative bacteria including *Salmonella typhi* by agar well diffusion method. It was seen that pelargonodin-3-galactose, cyaniding-3-glucose, quercetin, myricetin and gallic acid produced inhibitory zone of 13.8 ± 0.00 , 14.2 ± 0.45 , 14.0 ± 1.00 , 14.4 ± 1.51 and 18.4 ± 0.00 mm respectively. On comparison with our work, the zone size produced was less than the size produced by our three extracts. When the activity of all the isolated pure compounds was compared, out of the tested compounds, gallic acid showed the highest antibacterial activity against *Salmonella typhi* and the rest of organisms. The reason for antibacterial activity of all these pure compounds was attributed to their phenolic structure.

Minimum inhibitory concentration (MIC) of peel, pericarp and fruit extract was determined against forty five strains of *Salmonella typhi* by agar dilution assay (Table 6, 7, 8). Peel extract in this assay displayed the highest level of antibacterial activity (MIC 22mg/ml) against 45 strains of *Salmonella typhi*. Where as, pericarp and fruit extract showed MIC 24mg/ml and 64mg/ml respectively.

Prashanth D *et al* in India tested various extracts of pomegranate against a number of microorganisms. Antibacterial activity was evaluated by agar dilution assay. The petroleum ether, chloroform and methanol showed MIC of 12mg/ml. The water extract showed MIC of 25mg/ml against *S. typhi* which was comparable with the MIC range obtained from our study which was 16-22mg/ml for peel extract and 14-24mg/ml for Pericarp extract.

It may be concluded from this study that *Punica granatum* (pomegranate) has antimicrobial activity against *Salmonella typhi*. Evaluation of the antimicrobial activity can be more reliable if trials in animal models are taken.

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