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# Antibacterial Activity of Epicarp Extract of *Punica granatum* L. against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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## ABSTRACT

The ethanolic extract of *Punica granatum* (Family: Punicaceae) epicarp was studied for its antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The extract showed intense activity against MRSA. Serial chromatographic purifications by TLC, HPLC and $^1$HNMR offered two active compounds which were identified as tannic acid compounds which completely inhibited the growth of MRSA. The MIC value of these components was 25 µg/ml. These tannic acid components found in the epicarp of *P. granatum* might be useful in a phytotherapeutic strategy against MRSA.

**Keywords:** chromatography, $^1$HNMR, minimum inhibitory concentration, phytochemical, tannic acid
**Abbreviations: HPLC**, High Performance Liquid Chromatography; **MDRSA**, multi drug resistant *Staphylococcus aureus*; **MHA**, Muller Hinton agar; **MIC**, minimum inhibitory concentration; **MRSA**, methicillin resistant *Staphylococcus aureus*; **NMR**, nuclear magnetic resonance; **TLC**, thin layer chromatography; **VRSA**, vancomycin resistant *Staphylococcus aureus*

## INTRODUCTION

Staphylococci are Gram positive bacteria, 14 species of which are known to cause human infections, but the vast majority of infections are caused by only three of them. They are *Staphylococcus aureus*, *S. epidermidis* and *S. saprophyticus*. Of these, the most important species is *S. aureus.* Its main habitats are the nasal membranes and skin of warm blooded animals (Kuroda *et al.* 2001). It causes a range of infections from mild skin infections and food poisoning to life threatening pneumonia, sepsis, osteomylitis and infectious endocarditic (Projan and Novik 1997). The organism produces many toxins which are highly efficient at overcoming antibiotic effectiveness (Jevons 1961). Modern medicine faces a crisis as new stains of multidrug resistant bacteria are emerged threatening advanced treatments and intensive care. Every year nearly five million people die due to infections that do not respond to antibiotics, particularly Multi Drug Resistant *S. aureus* (MDRSA) infection. Drugs that have kept us safe thus far have begun to fail because bacteria are developing the ability to resist antibiotics. The application of more antibiotics against these bacteria has resulted in the development of multidrug resistance (Anonymous 2000).

In 1942, the year that penicillin G was introduced, resistant strains of *S. aureus* were found. Mitsuhashi *et al.* (1963) isolated tetracycline resistant *S. aureus* from clinical sources. Jevons (1961) in Great Britain isolated coagulase-positive, methicillin-resistant *S. aureus*. Vancomycin was the only antibiotic used against it, but in 1997, a vancomycin-resistant *S. aureus* (VRSA) strain was isolated (Makoto *et al.* 2001). At this critical juncture, we need to identify a new antibiotic to inhibit the growth of MDRSA. Flavonone containing plants belonging to the family Leguminosae are reported to possess antimicrobial activity against methicillin-resistant *S. aureus* (Tsuchiya *et al.* 1996). Extracts of medicinal plants in combination also showed antimicrobial activity against MRSA (Prakash *et al.* 2006). Mathebe *et al.* (2006) tested 21 plant species belonging to 14 families for antimicrobial activity against five different microorganisms, *Vibreo cholerae*, *Escherichia coli*, *Staphylococcus aureus*, *Sigella sonnei* and *Salmonella typhi* showed that most active extracts were those obtained from *Punica granatum* and *Indigofera daleoides*. Mathebe *et al.* (2006) further reported that the water extract of *Punica granatum* were equally active as organic extracts against bacteria such as *Staphylococcus aureus*, *Shigella sonnei* and *Shigella flexneri*. In the present study, the extracts of an important plant, *Punica granatum* L. belonging to the Punicaceae family has been selected.

Since preliminary trials of the present study showed good antimicrobial activity, ethanoloic extract of *P. granatum* epicarp was screened for antibacterial activity against MRSA and the isolation and purification of active phytochemicals were also attempted.

## MATERIALS AND METHODS

### Extraction, antibiotic disc preparation and assay

The epicarp of *P. granatum* L were collected and dried in room temperature for ten days. After ten days the dried epicarp was powdered with the help of mixer grinder under sterile condition. The powder was extracted using Soxlet unit, ethanol being the solvent. Antibiotic discs were prepared by sterile Whatman No. 1 filter paper (Sundararajan 1998). The discs were impregnated separately with the medicinal plant epicarp extract at concentrations of 5, 10, 20, 25, and 30 µg. Discs were dried in sterile condition and stored in sterile vials at 4°C. Muller Hinton Agar plates were prepared to check the antibacterial activity of *P. granatum* L epicarp extract. MRSA cultures were swabbed on MHA plates, and extract containing discs were placed on plates. Plates were incubated for 12 hrs at 35°C. Results were observed and recorded.

Received: 23 December, 2008. Accepted: 20 January, 2009.

Short Communication

## Thin layer chromatography

Thin layer chromatography was performed by the procedure of VWR, International Limited 2004. 25 g of Cellulose (TLC grade) powder was thoroughly mixed with 50 ml of sterile distilled water. The mixture was poured over a TLC plate maintaining the thickness of the layer at 250 mm and allowed to air dry for 1 hr. After drying, the later thickness reached 100 mm thickness. The cellulose-coated plate was activated at 120°C for 30 min. 0.1 µg of samples and Standard marker (tannic acid, Sigma) were properly loaded on TLC plate. The loaded plates were transferred to chromatographic jar containing Acetic Acid and distilled water (1:9 ratio). After running, the plate was air dried and sprayed with $NaNO_2$.

## High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography study was performed with Shimadzu SPD-6AV apparatus. Standard marker as tannic acid and fractions A, B and C were dissolved in methanol in the ratio of 1 mg/ml. 20 µl of marker, fractions A,B and C were injected into RP18 column of HPLC. Methanol and Water in the ratio of 2:1 was the mobile phase. The flow rate was 1 ml/mm.

## Hydrogen Nuclear Magnetic Resonance (HNMR)

Hydrogen Nuclear Magnetic Resonance was performed with a Bruker VC 200 USA 200 MHZ. The standard marker and fractions were dissolved in methyl $d^3$ alcohol-d. The standard marker and samples were transferred to cuvettes and kept inside the HNMR apparatus and the results observed.

## RESULTS AND DISCUSSION

*P. granatum* epicarp extract had good antibacterial activity. 25 µg disc produced a 13-mm zone against MRSA (**Fig. 1**). So the MIC value of *P.granatum* epicarp extract against MRSA was 25 µg/ml. The fractions produced were named A, B and C. The fractions were obtained in three different colors, i.e., pink, brown and brownish yellow respectively. The fractions showed different R*f* values (**Table 1**).

*P. granatum* epicarp extract fractions, B and C showed growth inhibition zone at 13 mm diameter against MRSA (**Fig. 2**). TLC plate fractions were analyzed in HPLC. Optical Density value was 7.000 at 310 nm using UV spectrophotometer. HPLC exhibited significant peak. All fractions were 100% similar to that of standard marker peak value. Standard marker peak value was 4.056. The value for fraction A 4.065, B.4.217 and C 4.424. In $^1$HNMR results confirm all fractions of the extract contain Tannic Acid-relevant components. $^1$HNMR results revealed standard marker, fractions A, B and C remaining in aromatic compound region.

Pharmacological investigations of medicinal plants have provided important dues for therapeutic approach to several pathologies as well as extremely useful tools for theoretical study of physiology and pharmacology. Essawi and Srour (2000) had submitted antibacterial activity of 15 plants against both Gram positive and Gram negative bacteria. But the present study was focused on communicable, dangerous oraganism such as methicillin-resistant *S. aureus*. Samy and Ignasimuthu (2000) reported that 20 plants showed antibacterial activities against several species of bacteria used for their assay. Most of the organisms such as *Bacillus subtilis* and *S. aureus* showed growth inhibition by *Cassia occidentalis* and *C. auriculata*. The present study is also similar to their study. Indian Tribal people used many Indian folklore medicinal plants against *B. subtilis*, *Escherichia coli*, *Klebsiella aerogens* and *S. aureus*. In their earlier studies using 10 mg plant extract discs of *P. granatum* to find antibacterial activity. In the current study, ethanolic extracts of *P. granatum* epicarp with 25 µg/ml was used to estimate growth inhibition of MRSA. *P. granatum* epicarp extract is found to be a very powerful drug to kill MRSA.

Caelli *et al.* (2000) reported that one nasal ointment contained 4% tea tree oil 5% tea tree oil body wash and 2% muporicin nasal ointment for the eradication of methicillin resistant *S. aureus* carriage. The whole fruit juice is good for treatment of heart ailments. *P. granatum* epicarp containing tannin-related components also can be used as nasal ointment and body wash for the removal of MRSA. Machado *et al.* (2003) reported that *P. granatum* leaf extract was used for the eradication of MRSA and MSSA. But the

**Table 1** $R_f$ values of *Punica granatum* epicarp ethanolic extract fractions.

| Standard marker | Extract | Fractions | $R_f$ value |
|---|---|---|---|
| Tannic acid | *Punica granatum* L | A | 0.2 cm |
| | epicarp | B | 0.3 cm |
| | | C | 0.45 cm |

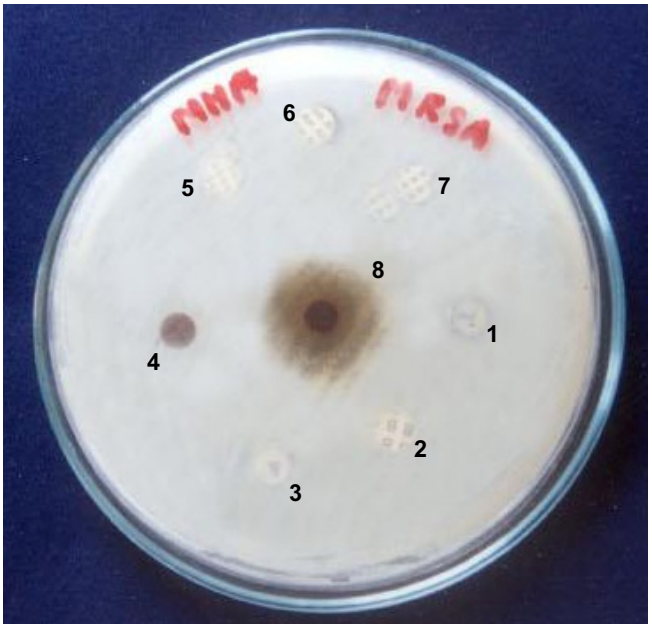


**Fig. 1 Antibacterial activity of *P. granatum* epicarp.** 1. Tetracycline, 2. bacitracin, 3. ampicillin, 4. doxycycline, 5. gentamycin, 6. novobiocin, 7. methicillin, 8. extract of *P. granatum* epicarp.

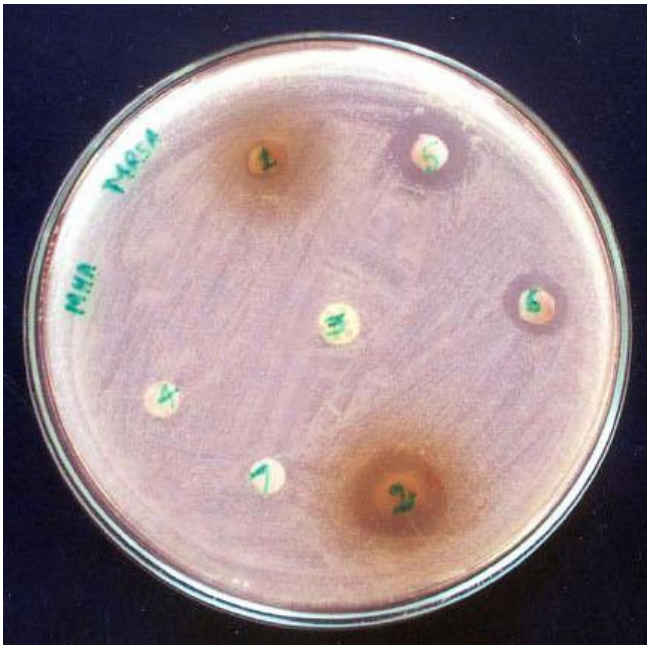


**Fig. 2 Antibacterial assay of *P. granatum* epicarp extract and TLC fractions.** 1. Standard, tannic acid, 2. extract of *P. granatum* epicarp, 3. methicillin, 4. fraction A, 5. fraction B, 6. fraction C, 7. control.

extract needs additional antibiotic chemicals as glycerol and vaseline. In the present study, *P. granatum* epicarp can eradicate the MRSA. Thus this extract is very efficient to kill the MRSA than the others. *P. granatum* was non toxic to human beings. The extract collected from *P. granatum* epicarp was already in use by ancestors for body wash. Gunther and Wagner (1996) reported that several commercial preparations could be made from pomegranates. But it needs addition of certain chemical substances to increase the quality. Astringents are prepared from highly economically important plants such as oak, catechu, nutmeg and aricanut. *P. granatum* epicarp is generally considered a waste material. Now it has been found as a useful material for the preparation of astringents.

TLC fractions A, B and C showed the presence of tannic acid. But fraction A showed no antibacterial activity against MRSA (**Fig. 2**). Preparative HPLC was useful for the isolation, identification and purification of compounds separated. Fernandez *et al.* (2005) reported that the common bean contained phytochemicals including phenolic compounds which can provide health benefits to the consumers. They used 100% methanol extract from seed coats that were subjected to different chromatic methods. But HPLC-MS gave a better separation of phytochemicals. Ding and Nie (2004) reported the identification of tannin component by HPLC-MS. The main components of epicarp extract of *P. granatum* were gallic acids, catechin, ellagic acid and hydrolysable ellagi. These components were separated by the use of HPLC-UV analyzer (Seeram *et al*. 2004).

$^{1}$HNMR studies showed different spectra depending on their location and adjacent molecules are surrounded by electron clouds which changes the encompassing magnetic field and thereby after the absorption frequency and confirming the presence of tannin. In the present study the presence of tannic acid chemical components in *P. granatum* epicarp was formed. NMR results showed in *P. granatum* epicarp extract peak compounds at aromatic region (6 ppm). Moore *et al.* (2005) reported the presence of purified polyphenol compound assigned to be 3, 4, 5, tri-*O*-galloyl quinic acid from the analysis of $^{1}$H and $^{13}$C one and two dimensional NMR spectra. They studied the purified tannins from the foliage of none species growing in the pygmy forest of the Northern Calfornia Coast with $^{13}$C NMR. A new ellagitannin, methyl (S)-flavogallonate along with known compounds, gallic acid, methyl gallate, ethyl gallate, 2, 3 di-*O*-[(s)-4,5,6, 4',5',6'-hexahydoxy biphenyl-2-2'-diyldicarbonyl]-(α/β) D-glucopyranose, vitexin, isovitexin, ellagic acid, flavogallonic acid and (α/β)-punicalgen were isolated from the leaves of *Terminalia myriocarpra* (Heurck Mazouk *et al.* 2002). But in the current study the suspected tannins isolated from *P. granatum* epicarp is yet to be identified.

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