

Original Research Article

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Evaluation of Antistaphylococcal Activity of Ellagic Acid Extracted from *Punica granatum* Fruit Peel on MRSA

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ABSTRACT

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Multiple drug resistance is always a challenge to any therapy. Phyto-compounds are one of the major sources of chemical for multiple drug resistance. The compounds used in the present study was extracted from pomegranate fruit peel and purified. Major compound extracted was ellagic acid. The extracted compound was tested for its antibacterial efficacy against wound isolates of *Staphylococcus aureus* and Methicillin resistant *S. aureus* (MRSA) using agar well diffusion method. Comparative study was done with gentamicin sulphate. Minimum inhibitory concentration of the ellagic acid was determined by microtube broth dilution method. The results showed that the biological activity of ellagic acid was lesser than gentamicin. Also, the minimum effective dose of ellagic acid was found to be 125µg/ml to MRSA, while the minimum effective doses of gentamicin was 30µg/ml.

Introduction

The immediate effect extended in saving the lives of patients, the medical community has to close their eyes to the serious effects caused by the antibiotics. Repeated and excessive use of antibiotics disturbs the person's own immunity, harms the beneficial bacteria inside body and can disrupt the internal ecology. In certain pathophysiological conditions such as pregnancy or infant diarrhoea the administration of antibiotics is harmful (Westphal *et al.*, 1994; Dancer, 2004; DuPont *et al.*, 2009).

When pathogens become resistant to first line antimicrobials, treatment has to be switched to second- or third-line drugs, which are almost always expensive. In many underprivileged countries, the high cost of such replacement drugs is unaffordable, therefore some diseases can no longer be treated in areas where resistance to first-line drugs is widespread (WHO, 2002). Multiple drug resistance (MDR) is a global issue that challenges the disease control and management. Hence, judicious use of

antibiotics is required, but acceptable strategies to achieve this goal and to address the situation is essentially need of the hour (Ganguly et al, 2011). *Staphylococcus aureus* is one among the foremost nosocomial pathogens and Methicillin-resistant *S. aureus* (MRSA) is a major cause of infections in hospitals (NNIS 2004; Drews et al., 2006). MRSA are also frequently resistant to most of the commonly used antimicrobial agents like the aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolone (Mandell, et al., 1995). Hence these bacteria are considered as a challenge to the available antibiotics and other drugs. Concurrently the search for novel and safer drugs against these bacteria bears prime significance. Hence It is necessary to search for a safer alternatives for antibiotics.

Since time immemorial human civilizations have used plants for medicinal purposes. In the present scenario of increasing drug resistance context medicinal plants are being re-assessed as models of antimicrobials mainly due to the development of MDR pathogens, emerging new infections, and the lack in the new antibacterial drugs in the pharmaceutical pipeline (Mahady, 2005). All the above points make a clear way for herbal antimicrobials.

P. granatum is globally distributed and is known as *delima* in Sanskrit and finds application in all traditional medical practices ie., *Ayurveda*, Folk, Homeopathy, Tibetan, Unani and *Sidha* medicines. Studies proved that pomegranate has a spectrum of medicinal properties including: bactericidal, antifungal, antiviral, immuno modulatory, antidiabetic activities etc. (Duman et al., 2009; Mahmood et al, 2010; Jain et al 2012) The extracts of plant parts are rich source of anthocyanidins- cyanidine, delphinidin,

phenolic acid like-caffeic acid, chlorogenic acid, tannic acids like gallic acid, ellagic acid and has curative effect on dysentery, AIDS, ulcers, skin lesions, cancer (Lansky and Newman, 2007; Reddy et al., 2007; Yehia et al, 2011). The presence of different kinds of polyphenolic antioxidants and commercial pomegranate juice has been shown to possess antioxidant activity three times higher than those of red wine and green tea (Gil et al., 2000, Singh et al,2002). The minimum inhibitory concentration of individual extracts of pomegranate rind and turmeric is recorded as the lowest concentration of drug (Suwipa et al., 2005)

The Phenolic compounds Present in plant parts have properties to denature enzymes (Furneri et al., 2002) but they can also bind to substrates such as minerals, vitamins and carbohydrates making them unavailable for microorganisms (Stern et al, 1996). Prevention of enterotoxin production from Staphylococcal species was reported by Braga et al (2005). Cell wall of the organisms absorbs phenols, resulting in disruption of the membrane structure and function (Hugo and Bloomfield, 1971). Due to the presence of large variety of phenolics and their biological activities and uses in medicines, pomegranate has been a plant of great interest and studied for its phenolic contents.

The present study deals with the extraction of total phyto compounds and purification of ellagic acid, a major component, from *P. granatum* peel, its characterization by IR, HPLC, & LCMS analysis and evaluation of the anti-staphylococcal efficacy of ellagic acid to clinical isolates of *S. aureus* and MRSA.

Materials and Methods

Fresh fruit of Pomegranate were picked

from orchards and brought to the laboratory. The fruits were washed, peeled; the rinds were cut into smaller pieces and then washed thoroughly with distilled water. It was then dried under sunlight. Pericarp was then kept in hot air (80°C) oven for 2-3 days. Dried Pericarp is then ground to get fine Powder. The standard of ellagic acid was procured from Sigma- Aldrich.

Isolation of Ellagic Acid

Pomegranate rind was processed as mentioned above, 100 gram of the same was taken and refluxed with 40 volume of methanol 3-4 times and methanol layer was concentrated. The residue was added with, 2 volumes of 30% Hydrochloric acid in methanol and refluxed for 3 hours and the residue was taken after filtration. Further it was washed with aqueous methanol. To improve the colour of the drug, residue was refluxed with 2 volume of Triethylamine for half an hour. The residue was filtered and washed with methanol to get 90% ellagic acid.

Characterizations of Ellagic Acid

Structural elucidation of ellagic acid was carried out by Infra-Red spectroscopy, Mass spectroscopy, NMR spectroscopy and High performance liquid chromatography

The Liquid Chromatography equipment (Thermo LCQ Advantage max) comprises a quaternary pump, an autosampler with 100µl loop and DAD detector. This is interfaced with an Ion trap mass spectrometer fitted with an APCI /ESI source operating in full scan mode to obtain fragment ion m/z. The condition optimized for collecting ellagic acid mass was using an APCI probe in negative ion mode with a capillary temperature of 200°C, discharge current (5 amp), vaporized temperature

(350°C), Sheath gas flow (80.0 ml/min) and, Auxiliary gas flow rate (10.0 ml/min)

Separation was achieved by HPLC-coupled to MS with Thermo BDS column having 250x4mm with particle size 0.45 micron. Mobile phase used was 0.1% orthophosphoric acid in water and 100% Acetonitrile. The gradient elution is from 0% to 100% for 40 minute.

Bacterial Strains and Growth Conditions

Staphylococcus aureus were collected and purified from Clinical isolates. The cultures were maintained on nutrient agar and stock cultures were maintained at 4°C. In all the experiments the evaluation of the antibacterial activity was screened with 18 hour old nutrient broth culture. The specimen was spread on blood, nutrient agar and inoculated into blood broth and were incubated at 37°C for 18 h. Identification was done based on colony characteristics, gram staining, tube coagulase test, and growth on Cystine-Lactose-Electrolyte-Deficient (CLED) agar and β hemolysis on sheep blood agar.

Evaluation of Antibiotic Sensitivity

Disc diffusion method was carried out to evaluate the antibiotic sensitivity test. Nutrient broth culture of *S. aureus* (18 hr., 0.5 McFarland Barium Sulphate standards) was swabbed on Mueller Hinton agar (MHA) plate in triplicate. Antibiotic discs were placed on agar with the help of a sterile forceps and pressed gently. The overnight incubated plates were checked for the presence of zone of inhibition around discs and finally the antibiotic sensitivity was assessed. Antibiotic discs evaluated included cefoxitin (10µg), methicillin (5µg), vancomycin (10µg), linezolid (30µg), tetracycline (30µg), penicillin G (10 units).

Evaluation of the Antibacterial Activity

In order to evaluate the antimicrobial activity of the isolated and purified phyto component from Pomegranate standard methods were adopted. Agar well diffusion method was employed for the evaluation of the activity (NCCL, 1999). A well-isolated colony of the MRSA, normal *S. aureus* and *S. aureus* ATCC strains from nutrient agar was inoculated in 5 ml of Mueller Hinton broth (MHB) in a test tube. MHB was incubated at 35°C, 2-6 hrs. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards, considered approximately equal to 1×10^8 CFU/ml. Wells of standard size (6mm) were incised on MHA equidistantly and the standardized broth culture was swabbed on separate agar media. Separate wells were loaded with 100µl of purified ellagic acid (100mg/ml), gentamicin sulphate (1mg/ml) as test and methanol and sterile distilled water as controls. Plates were incubated at 37⁰ C for 24 hrs and zone of growth inhibition were checked. Diameter of the zones were measured, consequently anti-staphylococcal activity was assessed. All the experiments were conducted in triplicates and an average value was taken.

Determination of Minimum Inhibitory Concentration (MIC) of the Ellagic Acid and Gentamicin Sulphate

The respective cultures of MRSA isolate and *S. aureus*, ATCC were grown in Mueller Hinton broth for 18 hrs and selected for the evaluation of MIC. Assay was performed in 96-well microtitre plates. 100mg of purified and crystalized ellagic acid was dissolved in methanol and the solution was heated up in a steamer to ensure complete dissolution in the solvent. These stock solutions were

diluted with MHB to a concentration of 1mg/ - 10mg/ml. Further 1:2 serial dilutions were performed by addition of culture broth to obtain 1.5 µg/ml- 1000 µg/ml. Gentamicin sulphate was also diluted in a similar way in sterile distilled water to obtain 2µg/ml- 1024 µg/ml. Inoculum density of the test organisms was adjusted to that of 0.5 Mc Farland standards. Broth was dispensed into wells of micro-titer plate followed by addition of the respective water extract and inoculum. Total volume of the assay system in each well was 200 µl. A methanol control was included in all assays and experiments were conducted in triplicates. Plates were incubated at 35 °C for 16-20 h and read at 600 nm in a plate reader (BIORAD 680).

Results and Discussion

Methanol extract of Pomegranate rind, subjected to HPLC analysis yielded a spectrum of phyto-compounds. The chromatogram reveal that the major compound obtained was ellagic acid marked at the peak at 9.96 min compared with the standard followed by a series of compounds between the retention times of 16-26 min (Fig. 1&2). This shows a range of polyphenols in the extract which contribute a major source of antioxidant reserve. The property of these compounds mark as key components which has major role in binding with the biomolecules when it is used as an antimicrobial.

The major compound in the spectrum was identified as ellagic acid which was isolated and purified to check the anti MSRA effect. The result showed that the compound has significant effect on the organisms evident with the zone of inhibition of 35mm with isolated *S aureus* and *S aureus*, ATCC while 21 mm with MRSA. The normal *S. aureus*, isolated from clinical and samples and

ATCC *S.aureus* highly sensitive to the compound while the MSRA is comparative resistant to it. MIC of ellagic acid was the lowest drug concentration wells that showed a prominent reduction (50%) in

growth/turbidity. When compared with antibiotics, the MICs of gentamicin sulphate were the lowest drug concentration wells that were optically clear without any visible growth/turbidity.

Table.1 Evaluation of Anti-staphylococcal Activity of Ellagic Acid on Different *S. aureus* species *Isolated from Hospital Samples, **Procured from ATCC *Methicillin Resistant *S. aureus*

Sl.No.	Name of the Organism	Anti-staphylococcal activity			
		Diameter of zone of inhibition (mm)		Minimum Inhibitory Concentration (MIC) (µg/ml)	
		Ellagic acid (10 mg)	Gentamicin sulphate (0.1 mg)	Ellagic acid	Gentamicin sulphate
1	<i>Staphylococcus aureus</i> *	35 ± 1.32	38 ± 1.53	125 ± 1.62	30 ± 2.01
2	MRSA*	21 ± 2.12	31 ± 2.82	125 ± 1.02	30 ± 2.22
3	<i>Staphylococcus aureus</i> ATCC**	35 ± 1.98	32 ± 1.65	125 ± 1.74	30 ± 2.16

Figure.1 Chromatogram of the Methanol Extract of Pomegranate Rind

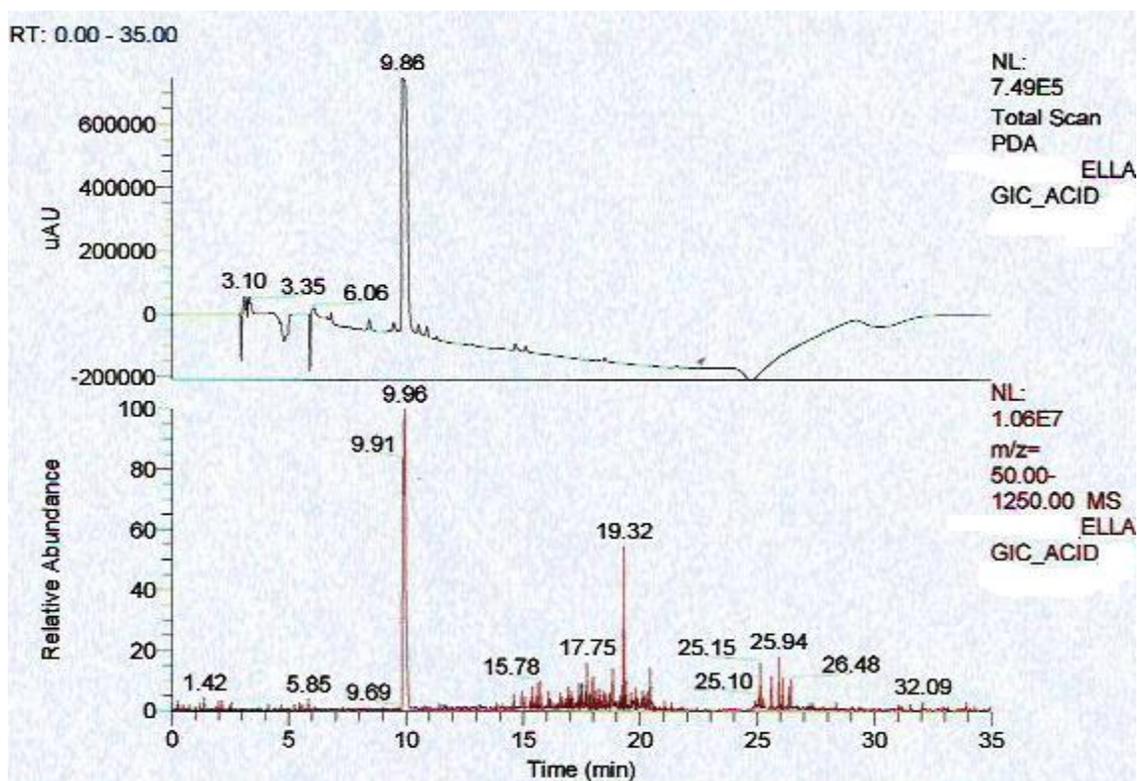
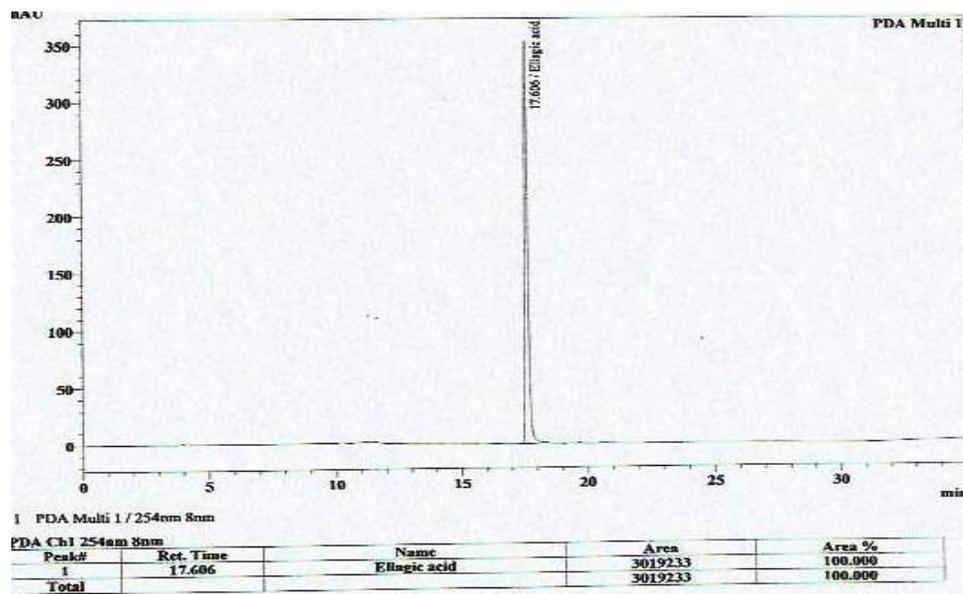


Figure.2 Chromatogram of Purified Ellagic Acid from the Rind of Pomegranate



Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of infections in hospitals and more recently in the community (Drews, 2006). MRSA includes those strains that have acquired a gene giving them resistance to methicillin and essentially all other β -lactam antibiotics which cause the same types of infections as other *S. aureus* strains (Lee, 2003). The prevalence of MRSA appears to be increasing at an alarming pace in India (Verma et al, 2000) and hence these pathogens bear prime significance as antibiotic resistant agents. Scientists are in search of novel drugs that can curtail the problems related to antibiotic resistant pathogens. Medicinal plants are considered to be the best source for compounds that can be used as drugs (Branen, 1975 Divyashree and Ravi, 2014) as many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as pharmaceuticals. The Tannin compounds from pomegranate are of great importance in developing drugs which can use as Anti-bacterials (Yehia et al 2011).

Previous reports on Pomegranate extract also indicated to have antibacterial activity against *S. aureus* (Ahmad and Beg, 2001). The phyto-component can be used for being a promising antimicrobial compound against both Gram-positive and Gram-negative pathogens, including oral pathogens in human (Loo et al 2010; Miguel et al 2010, Divyashree and Ravi, 2014, Lopes et al , 2014). *Punica granatum* peel extracts are used for treatment of respiratory diseases in the preparation of therapeutic formula. The anti-bacterial activity of *P. granatum* peels may be indicative of presence of metabolic toxins or broad spectrum antimicrobial compounds that act against both gram positive and gram negative bacteria. Ellagic acid, the major compounds from *Punica granatum* can be an alternate promising source to develop drugs against multiple drug resistant organisms

In conclusion, Methicillin-resistant *S. aureus* (MRSA) nosocomial pathogens are major causes of regular infections in hospitals. Multidrug resistance is an alarming situation

in the treatment for common pathogens. Natural compounds from plant source are studied and established to have preventive or therapeutic potentials. Ellagic acid, the major polyphenol compound of *Punica granatum*, isolated and purified was evaluated for the effect on MRSA. The study invariably proved that the ellagic acid is found to have significant antimicrobial properties against MRSA.

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