Antibacterial activities of gallic acid and gallic acid methyl ester on methicillin-resistant *Staphylococcus aureus*

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Abstract

Dried pomegranate peels were powdered and extracted by maceration in ethanol for 2 days at room temperature. The total ethanolic extracts were then successively partitioned to three parts to extract in a Soxhlet extractor with methyl acetate, -hexane and dichloromethane. The dried extracts were used to determine their antibacterial activity against clinical isolates of *Staphylococcus aureus* (MRSA and MSSA). All the peel extracts exhibited marked antibacterial activity. The antibacterial activity of methyl acetate extract inhibited the growth of all tested isolates, while dichloromethane extracts had no antimicrobial activity. Gallic acid (3,4,5 trihydroxybenzoic acid) is a naturally occurring polyphenol comprising the major hydrolytic product of tannic acid. Gallic acid (GA) and gallic acid methyl ester have been identified in pomegranate peels by the use of Nuclear magnetic resonance NMR (^{1}H, ^{13}C NMR). The ethanolic methyl acetate extract of pomegranate peels contained 100 and 10 mg/1g of GA and gallic acid methyl ester respectively. The overall results showed that the pure compounds of pomegranate peel extracts (Gallic acid (GA) and gallic acid methyl ester) have antibacterial activity.Minimum inhibition concentration (MIC) of Gallic acid and gallic acid methyl ester were demonstrated.

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1.Introduction

Pomegranate(*Punica granatum* L.), which belongs to the family Punicaceae, is commonly known as pomegranate, grenade, granats and punica apple, Supayang et al.,(2005). *Punica granatum* has been used extensively as a traditional medicine in many countries, Chidambara et al.,(2002). It is one of the important and commercial horticultural fruits which is generally very well adapted to the Mediterranean climate, Biale (1981) .

Medically beneficial compounds can be derived from the seed, juice, peel, leaf, flower, bark, and roots of the pomegranate. The broad scope and power of the pomegranate has been expanded recently with the discovery that the rind of the pomegranate contains antimicrobial activity that may be effective in the treatment of common hospital bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA).

Tannins are polyphenolic secondary metabolites of plants, which form hydrogen bonds in solutions, resulting in the formation of tannin-protein complexes, Sharma et al.,(1999). They are found in a large array of herbaceous and woody plants. Their molecular weights range from 500 to 3000 g $mole^{-1}$, Scalbert(1991); Chamkha et al.,(2002). Two groups of tannins are distinguished according to their structures: hydrolyzable and condensed ones, Regerat et al.,(1989); Huang et al.,(2005) Hydrolyzable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose, Bhat et al.,(1998) . They can occur in wood, bark, leaves, fruits and galls, Mueller-Harvey(2001).

Tannic acid is an important gallotannin belonging to the hydrolysable class, while catechin belongs to the non-hydrolysable class, Miranda et al.,(1996). Hydrolysable tannins are esters of phenolic acids and a polyol, usually glucose, Scalbert(1991); Chung et al.,(1998) annins have been reported to be bacteriostatic or bactericidal against *Staphylococcus aureus,* Chung et al.,(1993). Phenolic compounds occur in plants mainly as aglycones, glycosides or esters, or are bound to the cell wall. Acid hydrolysis can release the combined flavonoids and phenolic acids, Krygier et al.,(1982); Sosulski et al.,(1982).

The phenolic acids are either gallic acid in gallotannins, or hexahydroxydiphenic acid in ellagitannins. The hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid, Chung et al.,(1993) Gallic acid is the product of acidic or enzymatic hydrolysis of tannic acid, a readily available polyphenol in plants. Gallic acid (GA) and its dimeric derivative, known as ellagic acid(EA), exist either in the free or bound form as gallo-(GT) and ellagitannins(ET), respectively. These hydrolyzable tannins (HTs) are present in a rich variety of plants and are present in tea, red wine, fruits, beverages and various medicinal plants, Hatano(1995); Okuda(1995); Tanaka(1999). EA has been found to exhibit anti-mutagenic, antiviral, anticancer, antitumor and antioxidant properties, along with whitening of the

skin, Khanduja et al.,(1999); Stoner and Gupta(2001).

Gallic acid decarboxylases catalyze the second step in the degradation of the polyphenol tannic acid, Haslam et al.,(1961), Gallic acid seems to have anti-fungal and anti-viral properties, some ointments to treat psoriasis and external haemorrhoids contain gallic acid, Haslam et al.,(1961). GA has shown phyto-toxicity and antifungal activity against *Fusarium semitectum, F. fusiformis* and *Alternaria altternata,* Gallic acid http://www.phytochemicals.

Punica granatum, has been used extensively for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies, Ricci et al.,(2006); LinksHeber et al.,(2007).

In addition, *P. granatum* was reported to have antioxidant, Aviram et al.,(2004) Parmar et al.,(2008), antiatherosclerotic, Braga et al.,(2005); Parmar and Kar(2007) antibacterial, Braga et al.,(2005); Naz et al.,(2007) and antiviral, Lagrota et al.,(1986) properties. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties, Lansky(2007).

Punica granatum peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, scalds, diarrhea, dysentery and as an antioxidant, Singh et al.,(2002). In addition, it is reported that the extracts of *P. granatum* have antimicrobial activity against *Salmonella,* Lagrota et al.,(1986). However, to date, no studies regarding the antimicrobial activity of *P. granatum* peels have been conducted. Therefore, the aim of this work is to purify and characterize antibacterial active compound against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *S. aureus* from pomegranate (*Punica granatum*) fruit peels**.**

2.Materials and methods

2.1.Materials

2.1.Bacterial strains

Nine *Staphylococcus aureus* clinical isolates consisted of four methicillin sensitive *Staphylococcus aureus* (MSSA) and six methicillin resistant *Staphylococcus aureus* MRSA were used to study the antibacterial activities of Pomegrante peel extracts and pure compounds, Gallic acid and Gallic acid methyl ester.

2.1.2.Plant extraction materials

On kilogram of Pomegrante fruit peel powder was extracted as described by (Machado et al., (2003) Powdered by maceration in ethanol for 2 days at room temperature and the process was repeated twice. The total ethanolic extracts were concentrated in a rotational evaporator under reduced pressure and the residues were then successively partitioned to three parts to extract in a Soxhlet extractor with methyl acetate, n-hexane and dichloromethane. The solutions were completely evaporated to give the respective fractions. The methyl acetate fraction from the pericarp of Punica granatum fruit peels was applied to a XAD-16 resin (Sigma, St. Louis, USA) column and eluted with a continuous gradient of methanol in water from 5 to 100%. Further purifications over Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) by elution with methanol/water gradient from 0 to 50%, yielded a mixture of ellagitannins (PGF1). After further purification on preparative silica gel 60 (PF 254-366, Merck) TLC plates, two compounds were purified.

2.2. Methods:

2.2.1. Fractionation and identification of inhibitory compounds by nuclear magnetic resonance (NMR).

Nuclear magnetic resonance (NMR) is an analytical tool used by chemists and physicists to study the structure and dynamics of molecules, Webb (2011**).**

2.2.2.Antimicrobial potential of the plant extracts and phytochemicals

The antibacterial activities of the isolates on the different extracts were tested using a modified agar-well diffusion method, Machado et al.,(2003) . The bacteria cultures were grown in Brain Heart Infusion liquid medium at 35°C. A concentration of 10^6 cells /ml for each bacterial isolate was inoculated on the surface of Mueller-Hinton agar plates after 6 h of growth. A 100 µg/ml of each extract dissolved in DMSO was inserted simultaneously in a hole. vancomycin disk (30 µg) (Sigma Chemical Co) was used as the positive control and DMSO were used as the negative control. The plates were then placed in an incubator (Vision Co, Seoul, Korea) at 37°C for 24 h, after which the diameter of the inhibition zone around each disc was measured and recorded. Each experiment was performed in triplicate. The extracts that showed antimicrobial activity were later purified and tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample.

The Minimum Inhibitory Concentration (MIC) was determined by the agar dilution method in Mueller_ Hinton agar medium. Subsequently, bacteria (10^6 CFU/ml) were inoculated on the Mueller-Hinton agar surface. Purified compounds (gallic acid and gallic acid methyl ester) with concentrations ranging from 5-15 µg/ml were loaded after dissolved in 50% DMSO. Vancomycin disk (30 µg) was used as the positive control and DMSO were used as the negative control. The plates were then placed in an incubator at 37°C for 24 h, after which the diameters of the inhibition zone around each of the holes were measured and

recorded. Each experiment was performed in triplicate. The lowest concentration of gallic acid and gallic acid methyl ester, capable of inhibiting visible growth after 24 h of incubation at 37°C was then recorded as the MIC, Clinical and Laboratory Standards Institute (2001).

Table 1. Antimicrobial activity of Pomegrante fruit peel solvents against S. aureus (MRSA and MSSA)

S. aureus isolates	Pomegrante Extracts of			
	Vanco-mycin (30 µg/ml)	Methyl-actate (100 µg/ml)	Dichlor o methane (100 µg/ml)	Hexane (100 µg/ml)
MR1	29.33± 1.53	12.33±0.58	-	-
MR5	30±1.00	14.33±0.58	-	-
MR24	22.33±2,08	19.33±0.53	-	-
MR37	25.33±0.58	11.33±0.58	-	-
MR23	23±1,00	12.67±1.15	-	-
MR30	29.67±0.58	15.33±0.58	-	-
MS13	33.67± 0.58	15±1.00	-	-
MS14	29.67±o,58	17± 1.00	-	-
MS42	20±1.0	10.33±0.58	-	-
MS43	20.5±0.58	11±1.00	-	-

S. aureus MR= Resistant isolate to methicillin, *S. aureus* MS: susceptible to methicillin (Mean ±SD)

2.2.3.Statistical analysis

Data were analyzed statistically using SPSS software. The means were determined for significance at P<0.05 using LSD test.

***3.*Results and Discussion**

3.1. Preliminary evaluation of antimicrobial activity:

A concentration of 100 µg/ml methyl acetate of *P. granatum* peel extract was the best antibacterial activity against 10 strains of *S. aureus* by using agar well diffusion method in MHA as shown in Table (2).

Table 2. MIC to S. aureus (MRSA and MSSA) allic acid and Gallic acid methyl ester.

S. aureus isolates	MIC (µg/ml) of	
	Gallic acid	Gallic acid methyl ester
MR1	12.5	6.5
MR5	12.5	12.5
MR24	6.25	6.25
MR37	6.25	12.5
MR23	3.5	3.5
MR30	12.5	12.5
MS13	6.25	6.25
MS14	6.25	6.25
MS42	12.5	25
MS43	12.5	12.5

S. aureus MR : Resistant isolate to methicillin, S. aureus MS: susceptibil to methicillin(Mean ±SD)

The obtained results suggested that this class of compounds is responsible for the antimicrobial activity observed in this plan against MSSA and MRSA strains, Clinical and Laboratory Standards Institute (2000).

Results concluded that ellagitannins are the principal components responsible for the antimicrobial action of *P. granatum* against *S. aureus.* These polyphenols are known to form with proteins soluble complexes of high molecular weight. Thus, after being adsorbed, the polyphenols will react with the protein moiety of cell enzymes (oxido- reductases) in the cytoplasm and in the cell wall. They may also bind to bacterial adhesions and so, interfering with the availability of receptors on the cell surface, Machado et al.,(2003). The results presented here indicated that the natural products analyzed seemed to be a good choice for the development of new strategies to treat staphylococcal infections, including those caused by methicillin-resistant *S. aureus*. The hexane and Di chloro methane were inactive against all strains. This impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial effects of plant extracts using the agar diffusion method, Cowan(1999).

Tannic acid may work like a siderophore to chelate iron from the medium and make iron unavailable to microorganisms. Microorganisms growing under aerobic conditions need iron for a variety of functions, including reduction of the ribonucleotide precursor of DNA, formation of haem, and other essential purposes. Chung et al.,(1998) reported that the inhibitory effect of tannic acid on the growth of intestinal bacteria may be caused by its strong iron-binding capacity. Chung et al.,(1993) also reported that tannic acid inhibited the growth of all 15 of the bacteria tested, but gallic acid and ellagic acid did not inhibit any of them. They concluded that the ester linkage between gallic acid and glucose (to form tannic acid) was important to the antimicrobial potential of these compounds. In our study, the analysis of the growth inhibition activity using well diffusion method showed that the principal chemical constituents with antimicrobial activity were concentrated in the polar fractions of *P. granatum*, Avellaneda the major activity was detected in non-polar fractions. It is known that *P. granatum* is rich in hydrolyzable tannins, Hussein et al.,(1997) and this class of compounds has remarkable antimicrobial activity. The growth of fungi, bacteria, and viruses has been inhibited by tannins, Aqil et al.,(2005).The results also corroborate the previous antibacterial studies related to these two botanical species.

Tannin with gallate group has various physiological functions such as antibacterial, anti-allergic, scavenging free radicals, lowering blood pressure, serum and hepatic cholesterol concentrations and increasing fecal sterol excretion in rats with hypercholesterolemia, Aqil et al.,(2005)

Supayang et al.,(2005);Nostro et al.,(2006). Gallic acid(GA) and ellagic acid(EA) have been identified in pomegrante peels by the use of reversed-phase high-performance liquid chromatography(RP-HPLC) coupled with photodiode array detection (DAD).The ethanolic extract of longan peel contained 100 and 10 mg/1g peel of GA and EA, respectively. Afterheat treatment and acid hydrolysis, pomegrante peels had higher concentrations of GA and EA, contributing to more potent antioxidant activity. The results demonstrated rich sources of GA and EA in longan seed and mango kernel which might provide a novel source of these natural antioxidants.

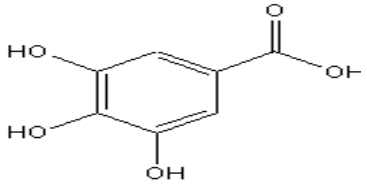
By using of Magnetic resonance spectroscopy (^{1}H, ^{13}C NMR), two compounds have been identified in pomegrant fruit peel extracts according to their peaks against those of standards. Analyses showed 1H-NMR spectra, nuclear magnetic resonance reference signal indicating an amount of offset (for Hedroginat 2.6) at 7.07 ppm. The analysis showed, signals indicated the existence of nucleus of a benzene ring with a carbonyl in^{13}C NMR and the information that was obtained revealed the chemical composition of the separated compound agreed with information published in scientific research globally, Haddock et al.,(1982) that the compound is an Gallic acid (Table 3 and fig. 1).

Table 3. (^{1}H, ^{13}C NMR) for gallic aid and gallic acid methyl ester

No.	Compound 1		Compound 2	
	C	H	C	H
1	121.9 s		121.1 s	
2 - 6	111.2 d	7.07 s	109.9 d	7.06 s
3 - 5	146.7 s		145.6 s	
4	140.8 s		138.5 s	
CO	168.7 s		166.8 s	
OMe			52.0 q	3.63 s

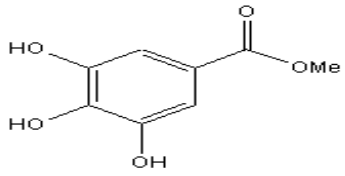
Analysis showed ^{1}H-NMR spectra, nuclear magnetic resonance (^{1}H, ^{13}C NMR) for each of hydrogen and carbon information was very similar to the first compound. In addition to the presence of signal indicating the existence of a methyl connected to a carboxyl group set and obtained information indicated the chemical composition of compound II was the Gallic acid methyl ester (Table 3 and Fig. 2).

Fig 1. The chemical structure of (1), gallic acid



1
Gallic acid

Fig 2. The chemical structure of (2) gallic acid methyl ester



2
Gallic acid methyl ester

3.2.Minimum inhibitory concentrations of GA and GAME

The MICs for antibacterial activity were established for the different extracts and fractions of *P. granatum* gallic acid and gallic acid methyl ester inhibit the growth of MRSA. The MIC of these compounds depended on the isolate by using the same 10 strains of MSSA and MRSA tested by the well diffusion method. The results presented in Table (2) showed lower MICs for gallic acid and gallic acid methyl ester that were ranging between 3.25 to 12.5 µg/ml in MHA for all tested strains (Table 2). The mode of regulation by phenolics at the bacterial proline dehydrogenase in the plasma membrane may be an important antimicrobial defense in plants and has consequences for disruption of critical energy metabolism of invading bacterial pathogen. The length of the alkyl chain has a key role in the elevation of susceptibility to-lactam antibiotics, Shibata et al.,(2005) This rationale could be used to design new antimicrobial strategies against *S. aureus* and other relevant bacterial pathogens, Kwon et al.,(2007).The overall results showed that the pomegrante peel extracts have antibacterial activity. Thus, it is extremely important to find new antimicrobial agents, new ways, that are effective for the treatment of diseases caused by drug-resistant bacteria including MRSA, Klevens et al.,(2007).

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