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Annals of Biological Research, 2013, 4 (5):75-87  
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## Pomegranate (*Punica granatum*) from ancient roots to modern life known with a potent antibacterial activity

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### ABSTRACT

Recently there has been an increasing interest in extracting relevant natural antimicrobial agents as potent as the chemical antibiotics to be used as an alternative approach for controlling growth of microorganisms. Pomegranate (*Punica granatum*) has taken great attention for its potent antimicrobial agent, in this study pomegranate homemade and market syrup (molasses) were tested for their antibacterial activity against 13 bacterial strains varying between gram positive bacteria such as *Staphylococcus aureus* ATCC 25923, *MRSA* ATCC 12498, *MRSA* ATCC 3345, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 29212, *Staphylococcus xylois* cilinical isolate, *Streptococcus pneumoniae* ATCC 6303, *Streptococcus viridians* clinical isolate and gram negative bacteria namely *Escherishia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603 and *Salmonella* sp. Agar well diffusion technique revealed the highest antibacterial activity of pomegranate syrup to be reported against *E.coli*, *S. aureus*, *S. xylois*, *Bacillus subtilis* respectively. Viable cell count and cell wall alteration were observed with time incubation assay particularly with *S. aureus* and *E. coli* after being incubated in pomegranate syrup at 37°C at different time intervals and examined by scanning electron microscopy (SEM). HPLC with total flavonoids and total phenolic content revealed that the major chemical component of pomegranate syrup is gallic acid to which potent antibacterial activity, decreasing viable cell count and structural alteration could be related.

### INTRODUCTION

Pomegranate, fig, olive grapes and dates mentioned in the Holy Quraan are considered as heavenly fruits of God indicating their beneficial effects.

Pomegranate (*Punica granatum*) is one of the oldest edible fruit which has a long history as a medicinal fruit extensively used in many cultures [16]. The edible parts of the fruit are consumed or used in the preparation of fresh juices, canned food and also in the production of tooth paste [14], [25], as well as in therapeutic formulas and cosmetics.

Since ancient times, pomegranate has been regarded as a “healing food” with a numerous therapeutic effects in several diseases [40]. It was used in eliminating parasites, treating aphtae, ulcers, diarrhea, acidosis, microbial infection and was also used as antipyretic [23], [24] and anti carcinogenic [17], [36], [20].

Nowadays, not only pomegranate is an important commercial fruit crop that is widely cultivated mainly in the Mediterranean parts of the world but it is considered as an alternative natural antibacterial agent for controlling growth of microorganisms.

The wide spread and over misuse of commercially available chemical anti-microbes led to the dangerous development of antimicrobial resistance of some human pathogenic microorganisms, threatening as such the public health. As a consequence, more emphasis was elucidated on medicinal plants known to have potent antimicrobial activity [19], [10] and have always been remedies for human health problems [37].

Pomegranate has been considered in many studies as having this antimicrobial activity [7], [9] [30]. In particular, there has been renewed global interest on the functional and nutritional benefits of pomegranate fruit [13].

The phytochemicals like tannins (gallic acid, ellagic acid) and flavonoids extracted from pomegranate revealed antimicrobial activity when assayed against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* MRSA and other harmful bacteria [31]. Braga revealed that pomegranate extracts inhibited not only the growth of *Staphylococcus aureus* but also the production of enterotoxins [9].

Many studies revealed that pomegranate is a fruit rich with polyphenols including ellagitanins, gallotannins, ellagic acid, gallic acid and catechins. These polyphenols exhibit various biological activities such as eliminating free radicals [28], inhibiting oxidation [4], antimicrobial growth [30], [7], [22] and decreasing the risk of cardiovascular diseases and some cancers [27].

The aim of this present study is to determine the in vitro antibacterial activity of homemade pomegranate aqueous extract syrup (molasses) compared to that found in the market against thirteen bacteria varying between gram positive and gram negative among which are human pathogenic antibiotic resistant strains such as *pseudomonas aeruginosa* ATCC 27853, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 12498, MRSA ATCC 3345 and *Salmonella* sp.

Mostly affected bacteria were selected for time alteration technique by incubating selected bacteria into the pomegranate syrup for different time intervals 24 and 48 hours at 37°C, after which bacteria were observed under the scanning electron microscopy for any changes that may occur on the cell wall which has to be studied further in the coming article on the genetic basis since few or no articles studied this alteration of the bacterial cell wall caused by pomegranate incubation assay. Chemical composition identifying mainly gallic acid present in homemade pomegranate syrup was performed using the HPLC.

## MATERIALS AND METHODS

### Collection of pomegranate

Fresh pomegranate fruit samples were collected from a local Lebanese fruit market. The fruits were cut into halves and seeds were separated from the skin, squeezed with water to release their juice. The juice was then brought to boiling to be transformed into pomegranate syrup or known as molasses widely used in the Mediterranean food. Fresh pomegranate homemade syrup together with the market pomegranate syrup were compared and studied.

Both homemade and market molasses were sterilized by filtration using the filter membrane unit of 0.45µm membrane size (Millipore, USA). Sterilized samples were kept in sterile eppendorf tubes at 4°C for further use.

### Bacterial Isolates

Bacterial strains namely *Staphylococcus aureus* ATCC 25923, MRSA ATCC 12498, MRSA ATCC 3345, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 29212, *Staphylococcus xyloso* cilinical isolate, *Streptococcus pneumoniae* ATCC 6303, *Streptococcus viridians* clinical isolate, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603 and *Salmonella* sp. were obtained from King Khaled Hospital, Microbiolo gy laboratory, Riyadh, Saudi Arabia. Each of

these tested microorganisms was freshly cultured on nutrient agar plates (Oxoid, USA) incubated at 37°C for 18- 24 hours.

### Preparation of Bacterial Suspension

Prior to susceptibility test, bacterial suspension of 0.5 MacFarland of each bacterium respectively was prepared in 5ml sterile nutrient broth (Oxoid, USA) test tubes to be inoculated on Muller Hinton agar plates (Oxoid, USA).

### Pomegranate syrup antibacterial susceptibility test

#### Agar well diffusion technique

Both pomegranate homemade and market syrups antibacterial activity was performed using the agar well diffusion technique [21]. Small inoculums of the bacterial suspension were spread with the help of sterile cotton swab on the surface of Muller Hinton agar plates. Inoculated plates were kept to dry, two equidistant holes were then performed by the use of a sterile cork borer of 6 mm diameter. Each well was loaded with 50µl of the two pomegranate syrups respectively. Plates were kept for 30 minutes at room temperature before incubation at 37°C for 18-24 hours, allowing as such the tested samples to diffuse into the agar.

The antibacterial activity was determined by measuring the inhibition zone in mm around the wells loaded with the fruit syrups. Standard antibiotic discs Ampicillin 10µg, optichin 10 µg, tetracycline 30 µg, vancomycin 30 µg, sulfamethoxazole 25 µg and oxycillin 1 µg related to each bacterium correspondingly were used as positive reference control, comparing as such the pomegranate bacterial activity with that of the chemically produced antibiotic discs. All tests were carried out in triplicates.

### Time Incubation Assay

Among the tested microorganisms in this study, four bacteria showing largest inhibition zone and being mostly affected with pomegranate syrups particularly *Staphylococcus aureus*, *Escherichia coli*, antibiotic resistant bacteria *Pseudomonas aeruginosa* and MRSA were selected to determine their cell viability and cellular morphological alteration when incubated in 1ml (v/v) pomegranate homemade and market syrups diluted in sterile distilled water (v/v,5ml/5ml) at different time intervals for 24 hrs and 48 hrs respectively at 37°C.

Prior to incubation, 0.1ml of pomegranate and each of the treated bacteria mixture was spread on the surface of nutrient agar plates to determine the cell viability and observe the structural changes that may occur on the bacterial outer layers. Plates were then incubated at 37°C for 18-24 hrs [32]. Colonies were counted to determine their viability and examined morphologically on the surface of the agar plates and further with scanning electron microscopy SEM for noting any cell wall alterations.

### Cell Viability Determination

Viable cell counting of the treated bacteria was performed on the nutrient agar plates and determined as number of cells/0.1ml. It was noted that lower bacterial count was obtained after 24 hrs of incubation in both homemade and market pomegranate syrups than with 48 hrs of incubation. Among the selected treated bacteria *Escherichia coli* showed the lowest number of cells after 24 hrs of incubation in the pomegranate syrups followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa* minor effect was observed on MRSA, this finding goes in agreement with Su *et al.*, 2012 [33] who stated that MRSA reduction depends on time as well as on the dose of the extract being used.

### Morphological Alterations

Not only bacterial cell viability was affected by the time incubation assay but also morphological changes was observed on the nutrient plates mainly on *E.coli* where the cells appeared to be larger, dry and bumped with an irregular margin, similarly *S. aureus* cells changed into dry cells having an irregular margin, *Pseudomonas* colonies appeared as dry and irregular; however, little effect was observed on MRSA. To gain insights on the bacterial mechanism of pomegranate syrups, scanning electron microscopy (SEM) was employed to further determine and observe the structural alterations that had occurred on the outer layers of the treated bacterial cells [34].

### Scanning Electron Microscopy (SEM)

After being morphologically described and for better understanding of the structural changes associated with pomegranate syrups treatment, selected colonies of the treated bacteria were cut into 5- by 10-mm pieces, fixed in 3% glutaraldehyde in PBS buffer for 1, followed by fixation in 2% osmium tetroxide for 1 h. The tissues were then dehydrated in ethyl alcohol, critical-point dried with carbon dioxide, mounted on aluminum stubs with silver paint

[35], vacuum coated with gold palladium alloy, and viewed by SEM at an accelerating voltage of 2 kV, at King Saud University, central Laboratory, Sciences College, KSA.

### Chemical Composition Analysis

#### HPLC

The chromatographic HPLC analysis of homemade pomegranate molasses was performed according to the method of Gil with slight modifications to determine mainly the percentage of gallic acid present in the sample being tested. Before injection, the sample was centrifuged (4 min at 5000rpm) and the supernatant was filtered through a 0.45µm Millipore filter unit (Millipore, USA). Injection volume was 50µl. An RP C18 Nucleosil 100 column was used for the separation of sample components. Mobile phase consisted of solvent A (2.5% v/v, solution of acetic acid in water) and solvent B (2.5% v/v, solution of acetic acid in methanol) at different ratios. Chromatograms were recorded at 280 nm. Gallic acid was quantified by comparing its highest peak related to other observed peaks. [15]

#### Total phenolic contents

The Folin-Ciocalteu method was used for the determination of total phenolic compounds with minor modification [21]. Briefly, 125 µl of standard gallic acid solution (0–0.32 mg/ml) or 125 µl of pomegranate home made syrup at 1 mg/ml in dimethylsulphoxide (DMSO) was added to 0.5 ml of distilled water and 125 µl of Folin-Ciocalteu reagent and then incubated for 10 min. Thereafter, 1.25 ml of 7% Na<sub>2</sub>CO<sub>3</sub> and 1 ml of distilled water were added into each sample. Then, all samples were warmed at 45 °C for 15 min. The absorbance at 765 nm was measured using a spectrophotometer. The concentration of total phenolic compounds was calculated from gallic acid standard curve and the data was expressed as mg of the gallic acid equivalent (GAE) per 1 g of dry weight sample. Fig.3

#### Total flavonoid contents

Total flavonoid content was evaluated according to a colorimetric assay with AlCl<sub>3</sub> with minor alteration [18]. Briefly, 250 µl of standard catechin solution (0– 0.32 mg/ml) or 250 µl of the home made pomegranate syrup at 1 mg/ml in DMSO was mixed with 1.25 ml of distilled water and then 75 µl of 5% NaNO<sub>2</sub> was added. After 10 min, 150 µl of 10% AlCl<sub>3</sub> 6H<sub>2</sub>O was added and the mixture stood at room temperature for 10 min. Next, 500 µl of 1 M NaOH and 275 µl of distilled water were added. Five minutes later, each sample was mixed and its absorbance at 532 nm measured using a spectrophotometer. The concentration of total flavonoids was obtained from a catechin standard curve and values were shown as mg of catechin equivalents (CAE) per 1 g of dry weight sample. Fig.4

## RESULTS

The therapeutic antibacterial effect of pomegranate aqueous seed extract pomegranate syrup against the fourteen tested bacterial isolates was studied and tabulated as the average of the inhibition zone measured in mm. (table 1)

**Table 1. Average inhibition zone measured in mm against the tested bacterial isolates indicating the antibacterial activity of both pomegranate homemade and market syrup. Highest antibacterial activity of pomegranate syrup was observed with the homemade mainly against *S. aureus*, *S. xylosus*, *E.coli*, *B. subtilis* and *S. pyogenes* with little effect on *MRSA*, *S. viridans* and *S. pneumoniae* respectively. Results were compared with the control standard antibiotic discs used for each of the tested bacteria.**

Bacterial isolates	Inhibition Zone in (mm)		
	Homemade pomegranate syrup	Market pomegranate syrup	Standard antibiotic discs (µg)
<i>Staphylococcus aureus</i>	26	26	Amp. (10) 29
<i>Staphylococcus xylosus</i>	23	21	Amp.(10)27
<i>MRSA</i>	22	18	Va (30) 25
<i>MRSA ATCC 3345</i>	20	15	24
<i>Streptococcus pyogenes</i>	23	19.5	OP (10) 25
<i>Enterococcus faecalis</i>	21	22	SXT(25) 27
<i>Streptococcus viridans</i>	15.5	17	OX(1)_
<i>Streptococcus Pneumoniae</i>	11	16.5	P(10) _
<i>Bacillus subtilis</i>	26.5	25.5	TE(30) 27
<i>Escherichia coli</i>	26	16.5	Amp.(10)_
<i>Pseudomonas aeruginosa</i>	16	14	OX(1)_
<i>Salmonella sp.</i>	11	—	SXT(25) 19
<i>Klebsiella pneumonia</i>	19	22	OX(1) 14

Amp.(10): Ampicillin, SXT (25): Sulfamethoxazole trimethoprine, TE: tetracycline, OP: Optichin, Va: Vancomycin, OX: Oxycillin, P: Penicillin.  
MRSA: methicillin resistant *Staphylococcus aureus*.



Fig.1 inhibition zone exerted by homemade and market pomegranate syrup respectively on *Bacillus subtilis* and *Staphylococcus aureus*.

The most potent antibacterial activity of pomegranate syrup was measured in mm and interpreted by the largest zone of inhibition observed mainly on *Staphylococcus aureus*, *Bacillus subtilis* and *E. coli* followed by *Staphylococcus xylosum*, *Enterococcus faecalis*, *MRSA* and *Pseudomonas aeruginosa*; however, little or no activity was observed on *Streptococcus pyogenes*, *S. viridans* and *Streptococcus pneumoniae* in agreement with other findings [2], [9]. The highest antibacterial activity was exhibited by the homemade pomegranate syrup rather than the market pomegranate syrup. Synergistic effects were observed when standard antibiotic discs were used on the total bacterial strains being tested particularly with *Staphylococcus aureus* and *Bacillus subtilis* [12].

#### Time incubation results

The time incubation assay results were best observed on *S. aureus* and *E. coli* affecting both the cell count and the structural morphology mainly after 24 hrs rather than 48 hrs of incubation with little alteration examined on *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* *MRSA*. These morphological changes were supported by the SEM where the treated bacteria appeared longer in size with filamentous margin as such different cell wall compared to the control untreated bacteria[34], [5]; however the mechanism of action is still not clear and should be examined and studied in our coming future work . (Fig. 2, fig.3)



Fig. 2 Effect of time incubation assay 24hrs at 37°C on *Escherichia coli*, bacterial cells are observed to be larger with an irregular margin and with a lower bacterial count.



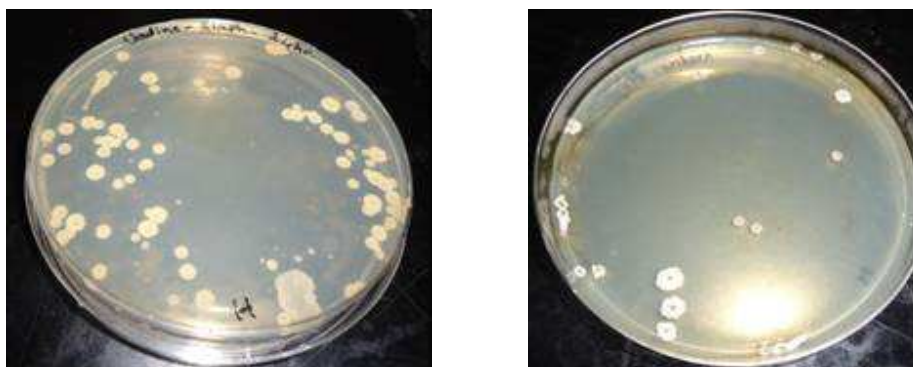


Fig.3 Effect of time incubation assay 24 hrs at 37°C on *Staphylococcus aureus*, bacterial cells are seen bigger than their normal size with an irregular margin.

Curves comparing the incubation time assay results:

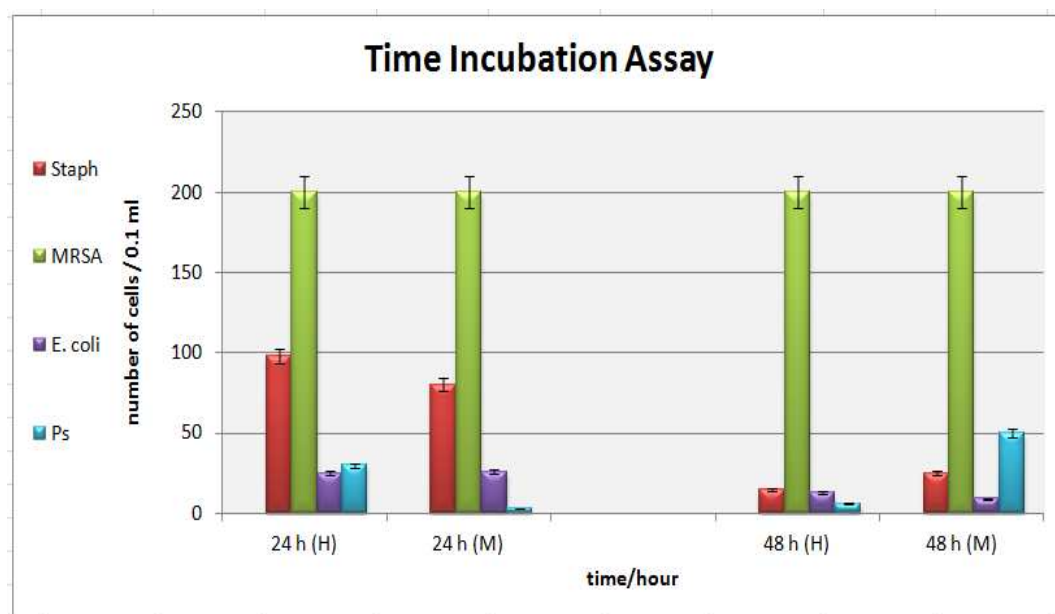


Fig. 4 Histogram comparing the time incubation assay effect on *S. aureus*, *E. coli*, *MRSA* and *P.aeruginosa*. Lower cell count is observed with *E. coli*, *S. aureus* and *P. aeruginosa* respectively.

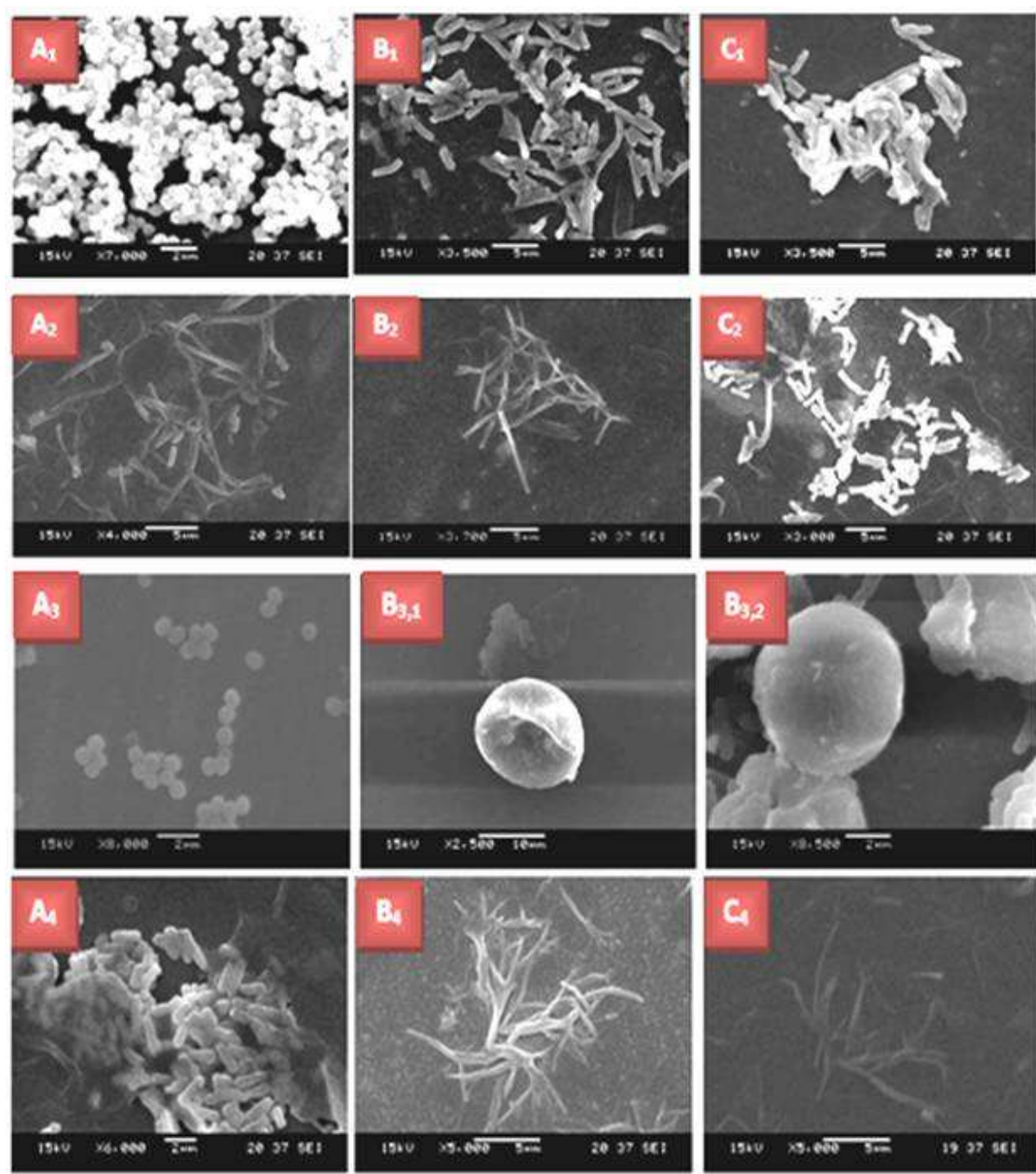


Fig.5 SEM of the four selected treated bacteria after being incubated at 37°C for 24 and 48 hrs in pomegranate homemade syrup showing cell wall alteration giving new horizons for our coming work. Lane 1: *Staphylococcus aureus*; lane 2: *Pseudomonas aeruginosa*. Lane 3: MRSA ATCC 3345, and Lane 4: *Escherichia coli*.  
A: control, B: incubation for 24 hrs and C: incubation for 48 hrs.

### HPLC Analysis

HPLC analysis with total flavonoids and total phenolic contents revealed that the major constituent of pomegranate homemade syrup is the hydrolysable tannins, gallic acid in correlation with other findings (fig. 6, fig.7 and fig.8), to which the major antioxidant and consequently the most potent antibacterial effect is being related [6], [12], [2], [8] and [41]. It was well known that plant phenolics and flavonoids are highly effective free radical scavengers and

antioxidants. It has been reported that oxidizing biological material leads to a rapid burst of superoxide, hydrogen peroxide, and hydroxyl (OH) generated primarily because of the ionizing of water molecules [3] which then interact with biological target molecules causing lipid peroxidation, DNA damage and subsequently resulting in cell killing and mutations [1]. This was obviously observed and emphasized in this study for the first time, using the time incubation assay for 24 hrs and SEM which revealed alteration in the cell wall, the cell viability and even the size of the bacterial cells being tested. Our next coming work is to determine, mainly on the DNA basis, the major chemical constituent of pomegranate syrup, gallic acid mechanism of action on some of the tested bacterial isolates.

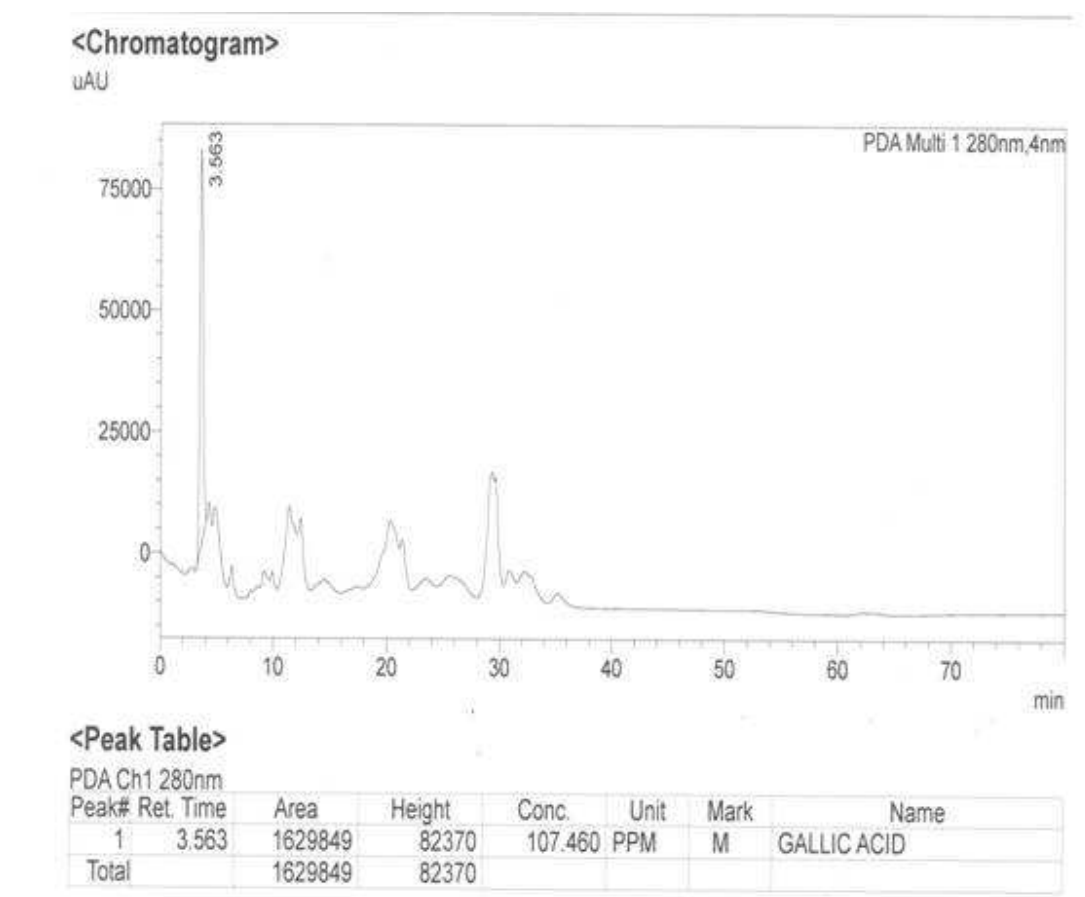


Fig. 6 HPLC chromatogram showing the highest peak refers to gallic acid, the major constituent of the home made pomegranate syrup sample being processed.



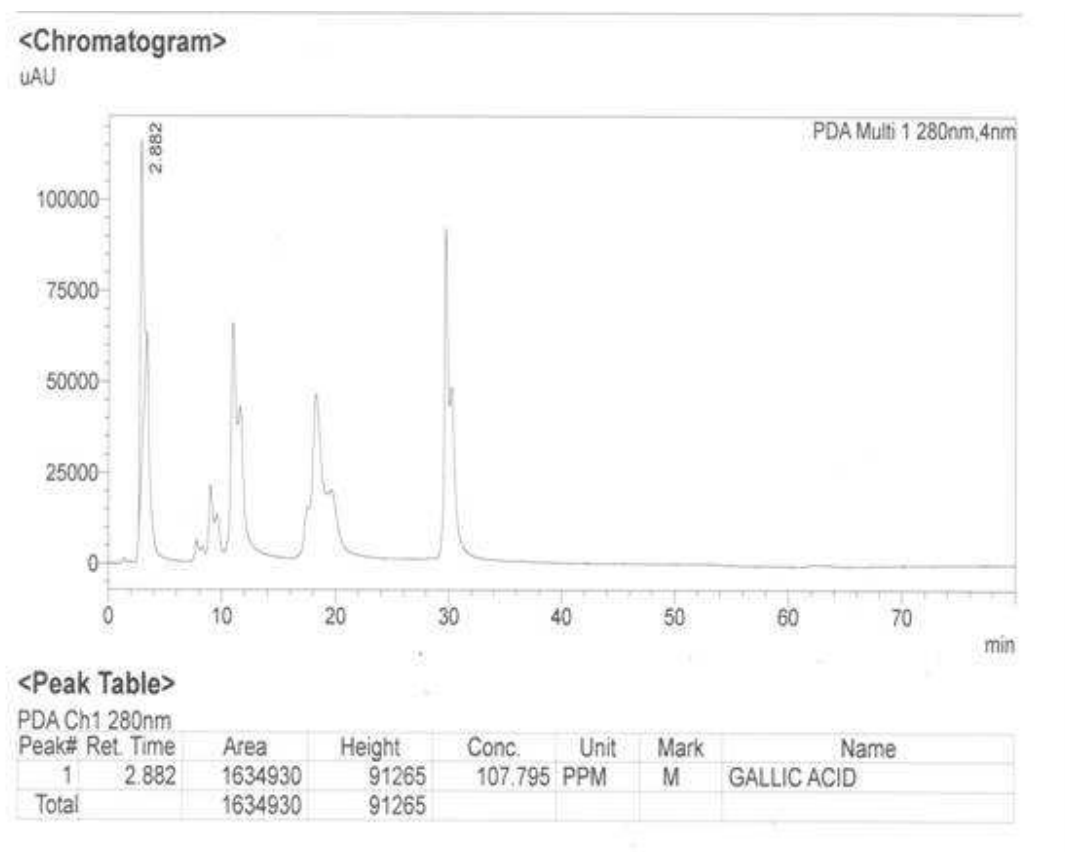
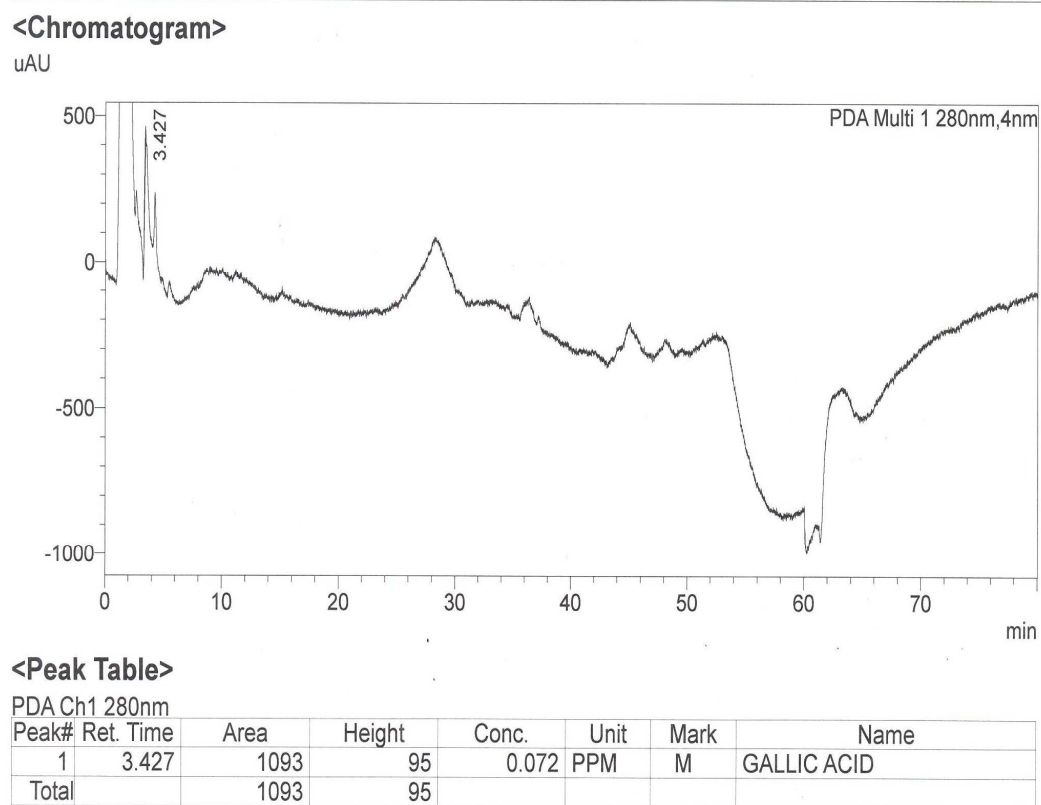


Fig.7 HPLC analysis chromatogram revealing the major constituent of pomegranate syrup being tested to be gallic acid indicated by the highest peak at 280 nm.



**Fig. 8** Pomegranate syrup HPLC chromatogram revealed the major peak observed is gallic acid as a major constituent (0.072x100/0.5ml= 14.4 ppm)

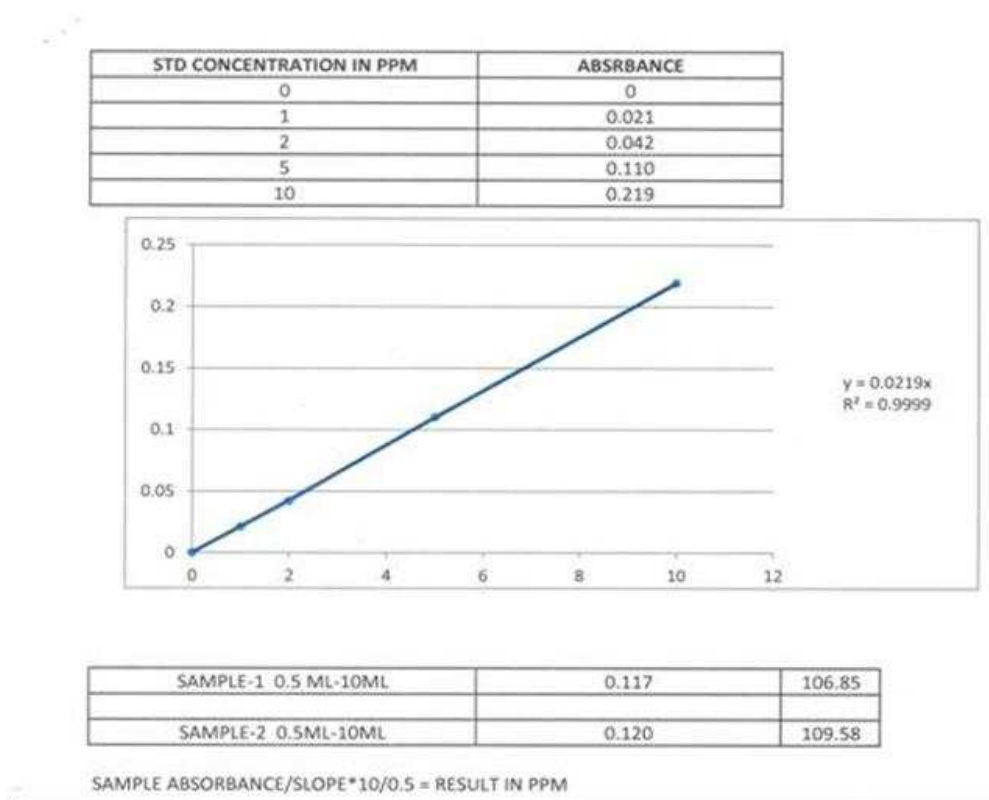


Fig.9 Total flavonoids slope showing the highest absorbance at 0.219 for 10ppm corresponding to gallic acid the main constituent of pomegranate syrup being tested.

## DISCUSSION

Several antibacterial mechanisms of plant derived extracts have been reported such as inhibiting the cell division, or damaging the cell walls of bacteria [32] and causing changes in microbial metabolism [5]. Several metabolites including tannins, flavonoids and other active substances have been previously associated with antimicrobial activity and extensively used in the replacement of the chemically produced antimicrobial agents [6].

Among these plants, *Punica granatum* or commonly known as pomegranate is well known from ancient times as a rich source of pharmacological properties due to its antioxidant, antimicrobial and antiproliferative activities. It was demonstrated that the potent antibacterial activity was mainly due to the presence of hydrolysable tannins and polyphenols specifically gallic acid in pomegranate extracts [2], [6], [39], [12] and [8].

Tannins may act on the cell wall and across the cell membrane by precipitating proteins. They may inhibit enzymes by oxidizing reagents and disturb co-aggregation of microorganisms [6], [2], [26] and [38]. Cowan [11] suggested that the antimicrobial properties of tannins resides in inactivating microbial adhesions, enzymes and cell envelope transport proteins, forming complexes with polysaccharides and modifying the cell morphology of microorganisms in agreement with our findings reported with the time incubation assay for 24 hrs in homemade pomegranate syrup where both cell viability and structural alterations were observed, giving new development strategies in the treatment of human pathogenic among which are antibiotic resistant where pomegranate syrup showed potent antibacterial activity against all tested bacterial isolates in this study. Braga observed that pomegranate extracts inhibit not only the growth of *Staphylococcus aureus* but also the production of enterotoxins [9]. *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *MRSA* were reported to be affected by pomegranate extracts [34], [6], [31], [32]; however, the mechanism of action is still unclear and should be studied in future work.

The attempt in this present work to reveal the antibacterial activity of pomegranate syrup and mainly how the bioactive compounds present in pomegranate extract could cause a drastic alterations in viable cell count together

with morphological changes after 24 hours of incubation at 37°C indicated that pomegranate could be used as an alternative potent antibacterial agent replacing as such the chemically used ones; however, the mechanism of action should be extensively studied for a better promising therapeutic usages of the ancient and heavenly fruit of God as mentioned in the Holy Quraan.

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