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Article

Lemon and Pomegranate Juices as Natural Inhibitors of srtA Gene Expression and Biofilm Formation in Clinical and Environmental Staphylococci

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Abstract: This study was conducted to examine the antibacterial and antivirulence effect of lemon juice and pomegranate juice on Staphylococcus aureus and Staphylococcus haemolyticus. Sortase A (SrtA) gene expression change, biofilm formation assay, and minimum inhibitory concentration (MIC) were used for inhibition kinetics grading. Fourier transform infrared spectroscopy (FTIR) also was performed to see presence of useful groups in organic activities. Based on the results, both juices resulted in strong bactericidal activity, such that pomegranate juice exhibits greater inhibition than lemon juice. S. haemolyticus was converted to a more sensitive phenotype than S. aureus, suggesting that susceptibility is different for certain bacterial species. The biofilm production of both extracts decreased significantly based on decreased optical density and almost negative biofilm phenotype. Also, gene expression analysis (SrtA) revealed a significant reduction in gene expression, indicating its significance in important adherence and biofilm processes. The presence of biologically viable purpose-built companies was investigated by means of FTIR assessment. These clusters interact with bacterial cell systems and the gene alters the pathways to provide the biological results that have been observed. In conclusion, lemon and pomegranate juices showed potent bactericidal and antivirulence habitat by inhibiting biofilm formation and suppressing SrtA gene expression These effects imply that they may provide ability herbal sources to be used within improving adjuvant treatment strategies in contrast to Staphylococcal infections.

Keywords: Pomegranate juice; Lemon juice; Biofilm inhibition; Staphylococcus aureus; Sortase A.

Introduction

The most outstanding clinical pathogens on the international scene, staphylococci can serve everything from minor skin infections to severe, occasionally fatal septic disease Methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus* (MRSA) are examples of multidrug-resistant *S. aureus* (MRSA) are examples of increased morbidity and mortality[1][2][3].

The ability of these microorganisms to form biofilms and express virulence elements are important techniques that help them survive and promote resistance to tablets and the immune machinery[4][5]. Surface proteins that promote attachment to host cells and biofilm development are generally responsible for their pathogenicity[6][7]. A critical component of this technique is Sortase A (SrtA), which promotes early adhesion and biofilm formation through the binding of proteins to LPXTG sequences to the bacterial mobile wall, so targeting this enzyme is a potential therapy that seeks to reduce pathogenicity as opposed to directly killing the microbe[8][9].

In this regard, studies show that lemon and pomegranate juice contain bioactive substances, especially flavonoids, together with Naringenin and ascorbic acid, which have antioxidant, anti-inflammatory and antibacterial properties. These compounds may inhibit *SrtA* by interaction with the active site affecting the enzyme's functional expression, leading to reduced biofilm formation and virulence factors[10][11]

Lemon juice also contains active functional groups such as carboxylic, carbonyl, and alkyl acids, which may contribute to enzyme inhibition through direct interaction with its active site[12]. Subsequently, pomegranate juice is characterized by the presence of several functional groups, including alcohols, alkanes, carboxylic acids, and ethers, which can additionally better inhibit through hydrogen bond formation and hydrophobic interactions, resulting in enhanced efficacy of inhibitory activity[13][14][15].

By evaluating their impact on biofilm formation and *SrtA* gene expression, the current study aimed to determine the antibacterial and antivirulence effects of lemon and pomegranate juices against *S. aureus* and *S. haemolyticus* and to explore their potential use as promising natural sources as adjuvant agents in treating infections associated with these bacteria.

Methodology

Staphylococcal Strains:

The *Staphylococcus* strains **(***S. aureus* & *S.haemolyticus*) used in this study were previously isolated from clinical samples and had been preliminarily identified by conventional methods and confirmed by PCR method targeting the 16S rRNA gene at the Department of Biology, College of Science, University of Mosul.

Preparation of Lemon and Pomegranate Juices

Fresh lemons (Citrus limon) and pomegranates (Punica granatum) were obtained from local markets and carefully selected to ensure that they are free from any visible damage or destruction, fruit turned thoroughly washed down and passed through tap water and then again sterilized with 70% ethanol for one minute. It was then rinsed with sterile distilled water to remove any remaining disinfectant. The lemon was peeled before juicing, while the pomegranate was manually seeded under sterile conditions. The juice was extracted using sterile juicer inside a laminar flow chamber to maintain sterility. The extracted juice was initially filtered through sterile gauze to remove coarse particles and then filtered again using Whatman No. 1 filter paper to obtain clearer solution. To ensure complete sterilization, the juice was sterilized again by membrane filtration using a 0.22-µm filter. Finally the aseptic prepared juice samples were transferred to sterile tubes and stored at 4ºC until later use, following standard laboratory procedures for preparation and sterilization of plant extracts[16].

Fourier Transform Infrared (FTIR)

The chemical composition of lemon and pomegranate juice extracts was analyzed using FTIR spectroscopy (Bruker , USA) to identify the major functional groups of bioactive phytochemicals presents in samples

Determination of minimum Inhibitory Concentration (MIC) and Sub-MIC concentration

The MIC of lemon and pomegranate against *Staphylococcus* strains was determined by using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Two-fold serial dilutions of each compound (500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9, and 1.95 mg/mL) were prepared in Mueller-Hinton broth and inoculated with 1.5×10^8 CFU/mL of the test strains. Dishes were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest concentration completely inhibiting visible growth. Sub-inhibitory concentrations (Sub-MICs) were

defined as concentrations below the MIC that allowed partial growth and were used in subsequent experiments**[**17][18]

Effect of Lemon and Pomegranate Juice on Biofilm Fourmation.

Sub-MIC concentration of lemon and pomegranate juice was tested to determine their effect on *Staphylococcus* biofilm formation using the 96-well microtiter plate assay. Bacterial suspensions (1.5×10^8 CFU/mL) were incubated with Subinhibitory concentration of each compound at 37 °C for 24 h. The wells were washed with PBS, stained with 0.1% crystal violet dye, and then dissolved in 95% ethanol. The amount of biofilms formed was measured by absorbance at 630 nm. The experiment included Control wells free of the compounds[19][20].

Effect of Lemon and Pomegranate Juice on *SrtA* gene expression,

The effect of Sub-MIC concentrations of lemon and pomegranate juice on *SrtA* gene expression in *Staphylococcus* strains was evaluated using quantitative real-time Polymerase Chain Reaction (qRT-PCR). Bacterial cultures (1.5×10^8 CFU/mL) were incubated with each compound at its respective sub-inhibitory concentration (Sub-MIC) for 24 h at 37 °C. Total RNA was extracted using a commercial RNA isolation kit (Tras,Zol upplus RNA kit, Transgen, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using a reverse transcription kit (EasyScript® First-Strand cDNA Synthesis SuperMix Kit, Transgen, China).Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using *SrtA*-specific primers, and gene expression levels were calibrated use *GAPDH* gene as a reference gene. The primers used were as follows:

SrtA gene: Forward primer 5'-GTGGTACTTATCCTAGTGGCAGC-3', Reverse primer 5'-GCCTGCCACTTTCGATTTATC-3' . *GAPDH* (reference gene): Forward primer 5'-CAGCTCGTGTCGTGAGATGT-3', Reverse primer 5'-CGTAAGGGCCATGATGACTT-3' . Relative gene expression was calculated using

the 2^−ΔΔCt method. All experiments included vehicle-free control cultures as a baseline for comparison.

Statistical analysis:

All experimental results were subjected to statistical analysis using one-way analysis of variance (ANOVA). Mean ± standard deviation (SD) is used to display the data. A statistically significant p-value was defined as less than 0.05.

Results

Table 1 show the MIC and Sub-MIC values of **Lemon and Pomegranate Juices** against the tested *Staphylococcus* strains which exhibited a pronounced antibacterial activity against *Staphylococcus aureus*, with comparable inhibitory observed in both environmental and pathogenic strains. In contrast, it is activity against *Staphylococcus haemolyticus* showed strain –depended variation, with pathogenic strains demonstrating greater susceptibility than environmental isolates. With regard to pomegranate juice, the findings revealed that most potent inhibitory effect was recorded against the environmental strains of *Staphylococcus haemolyticus* , whereas relatively lower activity was observed against the remaining tested strains.

Table 1. MIC and Sub-MIC values of Lemon and Pomegranate Juices against Staphylococcus strains.

Staphylococcus strains		**Lemon Juice**		**Pomegranate Juice**	
Source	Species	MIC (mg/mL)	Sub-MIC (mg/mL)	MIC (mg/mL)	Sub-MIC (mg/mL)
Clinical	*S. aureus*	31.25	15.62	7.8	3.9
	S. haemolyticus	15.62	7.8	31.25	15.62
Environmental	*S. aureus*	31.25	15.62	15.62	7.8
	S. haemolyticus	62.5	31.25	3.9	1.95

FTIR evaluation of lemon juice, Figure 1, shows about five-six distinct concrete units, including hydroxyl (-OH), carbonyl (C=O), alkane (-CH2/-CH3), and bond (C-O), with amine (-NH) probable Signals below 500 cm^{-1} Inorganic or halo-C bonds). They also show. This spectrum shows the chemical composition of lemon juice, which is rich in phenols, citric acid and organic acids, and carbohydrates. FTIR analysis of pomegranate juice in Figure 2 reveals approximately four–five dominant functional occupations: hydroxyl agent (–OH), carbonyl compounds (C=O) indicating the presence of phenolic alcohols associated with hydrogen bonds regularly associated with organic acids and phenolic compounds; and (C–O) bonds, which indicated the presence of sugars, carbohydrates, and complex phytochemicals in the juice. This spectral model shows the rich composition of pomegranate juice, along with phenolic compounds, sugars, and other herbal bioactive compounds.

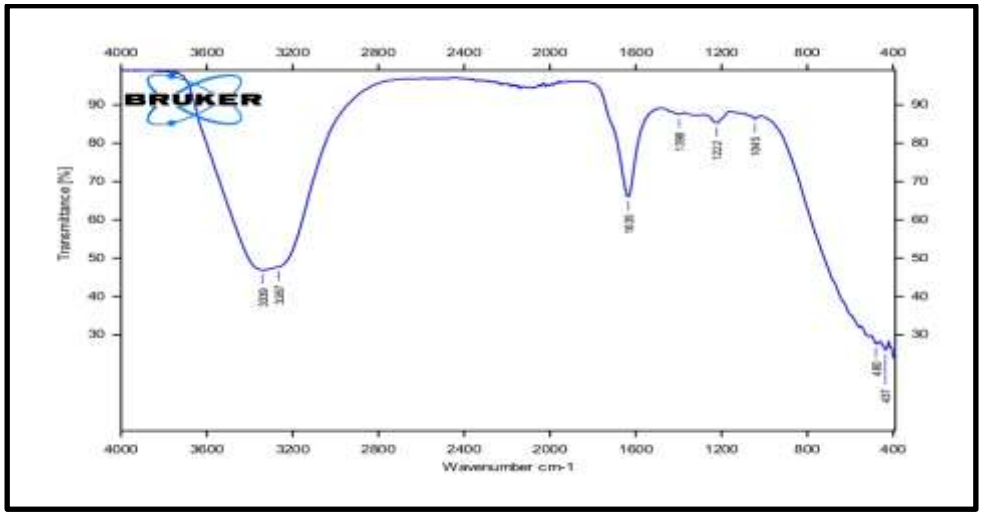

Figure 1. FTIR Analysis of Lemon Juice.

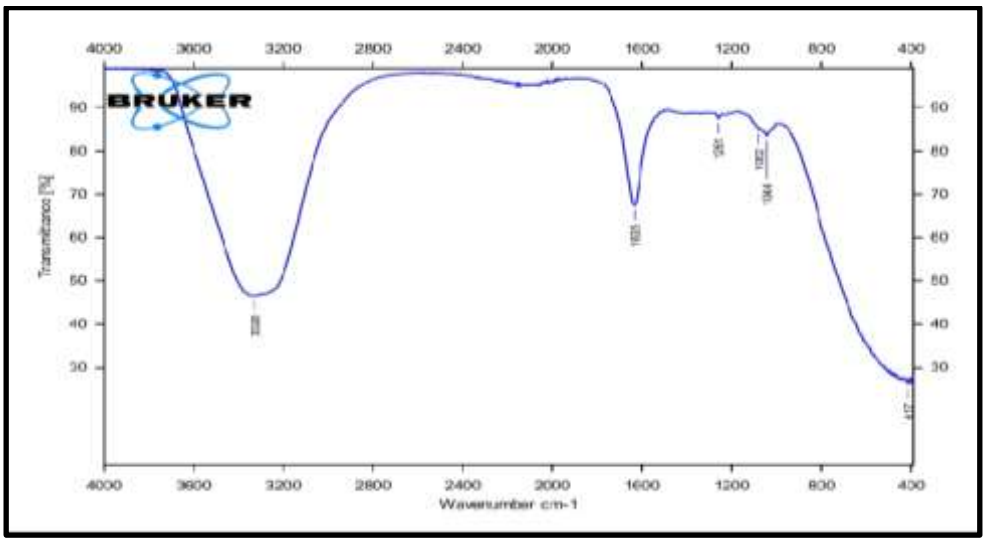

Figure 2. FTIR Analysis of Lemon Juice.

Table 2 shows the effect of **Lemon and Pomegranate Juices** on biofilm formation by *Staphylococcus* strains. The control groups exhibited biofilm formation, with *S. haemolyticus* showing moderate formation (+) and *S. aureus* showing strong formation (++). In contrast, treatment with **Lemon and Pomegranate Juices** significantly inhibited biofilm formation in both strains, as indicated by low optical density values and negative classification (−).

Table 2. Effect of Lemon and Pomegranate Juices on biofilm formation by Staphylococcus strains using the microtiter plate assay.
OD: Optical density at 630 nm. Biofilm formation: OD ≤ 0.1 (−), OD > 0.1 (+), OD ≥ 0.2 (++).

Staphylococcus strains		**Control**		**Lemon Juice**		**Pomegranate Juice**	
Source	Species	OD630	Biofilm	OD630	Biofilm	OD630	Biofilm
Clinical	*S. aureus*	0.126	+	0.024	_	0.29	_
	S. haemolyticus	0.125	+	0.06	_	0.039	_
Environmental	*S. aureus*	0.247	++	0.02	_	0.04	_
	S. haemolyticus	0.189	+	0.02	_	0.01	_

Table 3 shows, using qRT-PCR, how lemon and pomegranate juice affect bacterial SrtA gene expression of S. aureus and S. haemolyticus both bacterial strains significantly reduced SrtA gene expression with both treatments compared to the control group ($p < 0.05$).

Table 3. Effect of Lemon and Pomegranate Juices on SrtA Gene Expression in Staphylococcus Strains.

Staphylococcus strains		Treatment	*SrtA* **gene**	GAPDH gene	ΔCT	ΔΔCT	Folding
Source	Species						
Clinical	*S. aureus*	Control	28.9	22.3	6.6	0	1
		Lemon Juice	29.9	22.5	7.4	0.8	0.574349177*
		Pomeg.Juice	30.2	22	8.2	1.6	0.329876978*
	S. haemolyticus	Control	30	22.3	7.7	0	1
		Lemon Juice	31	22	9	1.3	0.406126198*
		Pomeg.Juice	31.3	22.3	9	1.3	0.406126198*
Environmental	*S. aureus*	Control	31.2	22	9.2	0	1
		Lemon Juice	32.9	22.4	10.5	1.3	0.406126198*
		Pomeg.Juice	32.9	21.9	11	1.8	0.287174589*
	S. haemolyticus	Control	31.6	22.4	9.2	0	1
		Lemon Juice	33.6	22.2	11.4	2.2	0.217637641*
		Pomeg.Juice	33.3	21.9	11.4	2.2	0.217637641*

***Significant differences ($p < 0.05$)**.

Discussion

Our study demonstrated that both lemon juice and pomegranate juice exhibit strong antibacterial and anti-virulence activities against *Staphylococcus aureus* and *Staphylococcus haemolyticus*. Based on MIC values (Table 1), pomegranate juice showed superior inhibitory activity compared to lemon juice. Notably, *S. haemolyticus* evolved into a more susceptible form, possibly due to differences in cell wall architecture, membrane permeability and intrinsic resistance mechanisms[21][22].

Both extracts inhibited biofilm formation by a major factor (shown in Table 2), with lower optical density and a negative biofilm phenotype. Biofilms are very beneficial for bacterial endurance, immune evasion, and antibiotic resistance, and their inhibition of these extra-natural elements shows promise. Adhesion-cell harvesting method, the extent of which varies according to the origin of isolation and biological composition[23][24]. It was determined that isolate resources exhibited a clean difference and that the clinical isolates showed a superior biofilm-forming potential relative to the environmental ones. Environmental isolates were more attentive to plant extract treatment in the evaluation and clinical isolates revealed greater their resistance to virullect, refpass resistance[25][26].

At the molecular level, gene expression analysis (Table three) showed significant downregulation of SrtA in each treatment arm, stronger effect of pomegranate juiceSortase A is the main enzyme responsible for anchoring basal proteins to the LPXTG motif, which promotes adhesion and ear. Its inhibition is therefore associated with attenuation of biofilm formation and impaired bacterial colonization. These effects are closely linked to the phytochemical composition discovered in FTIR analysis (Figure 1 and a couple), which unquestionably confirmed the presence of hydroxyl (-OH), carbonyl (C=O), phenolic, aromatic, and C-O advantageous units; transcriptional function regulation; bacterial enzyme regulation; and enzyme gene expression pathways[27][28].

Mechanistically, SrtA suppression prevents surface proteins from attaching to the bacterial cell wall, thereby hindering the initial adhesion and microcolony formation, which are critical early stages in biofilm growth[29][30][31]. These results in less bacterial colonisation, reduced pathogenicity, and increased susceptibility to antimicrobial agents and host immune responses[32][33].

Conclusion

Lemon and pomegranate juice have potent antivrulence and antibacterial properties that are mediated by decrease of *SrtA* gene expression and inhibition of biofilm formation. These findings demonstrate that a very potent supply of these plant extracts may exist, despite the fact that they are currently restricted to in vitro settings. Even while those extracts are appealing as supplemental medicinal agents, they have not yet been used with living organisms. In vivo studies are necessary to

confirm this and further explore pharmacokinetics, effectiveness, and the possibility of synergy with traditional antibiotics.

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