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Exploring Grapefruit Juice as an Antimicrobial Agent Against *Proteus mirabilis* Biofilms

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ABSTRACT

This present work tested the antimicrobial efficacy of grapefruit juice and its effect on biofilm-forming capacity and gene expression in isolates of *Proteus mirabilis*. Minimum inhibitory concentration (MIC) for grapefruit juice against different *P. mirabilis* isolates were estimated, signifying variable susceptibility among *P. mirabilis* isolates. The majority of isolates revealed an MIC of 50%, whereas other isolates had a somewhat lower MIC of 25%. In addition, fold change values, in particular of the *mrpA* gene associated with biofilm formation, were tested before and after treatment with grapefruit juice. Significant differences in fold change values were noticed among isolates. Yet, despite the overexpression of *mrpA*, grapefruit juice inhibits biofilm formation, representing the regulation of biofilm complication and the multifactorial impacts of antibacterial ingredients found in grapefruit juice. In conclusion, the present investigation settles the potential antimicrobial efficacy of grapefruit juice against *P. mirabilis*. More investigatory work is necessary for a better understanding of the mode of action and exploring the clinical applications of grapefruit juice in the treatment of urinary tract infections and other bacterial diseases.

Keywords: Biofilm, Proteus mirabilis, grapefruit, mpA.

I. INTRODUCTION

The species *Proteus mirabilis* resides in the gastrointestinal tract but can cause urinary tract infections (UTI) in immunocompromised patients or those that have urinary tract anatomical abnormalities, due to the microorganism's capability to form impenetrable biofilms on urinary catheters and in the urinary tract, resulting in repetitive infections and eventually antibiotic resistance [1]. Numerous of these studies have underscored the significant role of Mannose-resistant Proteus-like fimbriae (MrpA) encoded by the *mrpA* gene in stimulating *P. mirabilis* biofilm formation critical for a key factor in the urinary tract and catheter-associated infections [2]. Al-Hamdani and Al-Hashimy [3] also reported that the *mrpA* gene was detected in *P. mirabilis* isolates in a percentage of 80%. These studies cumulatively underscore MrpA fimbriae as being clinically relevant in *P. mirabilis* pathogenesis and emphasise the urgency of targeting these biofilm-associated mechanisms in order to develop an efficient treatment regimen and infection control strategy. With the worrying rise of multidrug-resistant strains of pathogenic microorganisms, there is an urgent need to evaluate alternative therapeutic strategies. This research focuses on natural compounds presenting antimicrobial activities such as fruit juices as potential adjunctive or alternative treatments to antibiotics [4].

Regarding the UTI caused by *P. mirabilis*, exploring the probable antimicrobial outcomes of grapefruit juice is

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specifically pertinent. Latest studies have underscored the promise of natural compounds, which include fruit juices, as alternative or adjunctive therapies for bacterial infections. Fruit juices, with their diverse array of bioactive compounds, provide a hopeful road for addressing antibiotic-resistant pathogens and reducing the selective stress driving antibiotic resistance [5, 6].

Grapefruit juice has numerous bioactive agents, embracing flavonoids and phenolic acids, which might be related to its antimicrobial compounds [7]. Research has tested grapefruit juice's capability to inhibit the growth of several bacterial species, including *Escherichia coli* and *Staphylococcus aureus* [8, 9]. Albeit, the precise results of grapefruit juice on *P. mirabilis* warrant more investigatory work. Knowing the capacity of inhibitory consequences of grapefruit juice on *P. mirabilis* growth and virulence should preserve huge medical implications. UTIs caused by *P. mirabilis* pose treatment-demanding situations due to the bacterium's adeptness in forming robust biofilms and its intrinsic resistance to many antibiotics [10].

Recognising novel treatment plans like grapefruit juice ought to revolutionize UTI management and antibiotic use. Testing grapefruit juice's effect on *P. mirabilis* biofilm formation and motility is important for developing powerful adjunctive. Investigating fruit juices as antibiotic alternatives is critical in the face of increasing antibiotic resistance [11].

As far as we aware, this is the first report testing the effect of grapefruit juice on biofilm formation and *mrpA* gene expression. However, for the aforementioned facts, the prevailing investigation aimed toward unveiling grapefruit juice's antibacterial properties towards *P. mirabilis* biofilm formation and reconnoitring its impact on *mrpA* expression.

II. METHODS AND MATERIAL

Preparation of grapefruit juice:

After thorough rinsing with distilled water and surface sterilized with 70% ethanol, the grapefruit fruit (*Citrus paradisi*) was cut into halves with the aid of a sterile knife, and their juice was extracted by the use of a juice extractor. The juice underwent slight non-stop drift pasteurization, ensuring a temperature beneath 85°C. Upon completion of this process, the samples have been cooled for no longer than one week before evaluation. Extensively, those juices maintained their natural turbidity, devoid of any additives or extra water. With a low pH, changes have been vital to mitigate potential inhibitory consequences on the examined isolates. To cope with this, the grapefruit juice turned neutralized by 1N NaOH solution until attaining a pH of 7 ± 0.2, simplifying the testing of its antibacterial efficacies [12].

Proteus mirabilis isolates:

Test isolates comprised fifteen *P. mirabilis* strains sourced from the collection maintained at the Microbiology Lab within the Department of Biology, College of Science, University of Baghdad.

Determination of the minimal inhibitory concentration of grapefruit juice:

The minimum inhibitory concentration (MIC) assay for grapefruit juice was carried out via the broth microdilution technique as described by the Clinical Laboratory Standards Institute [13] with slight adjustments, in brief.

Successive twofold serial dilutions of grapefruit juice starting from 50% to 0.78% had been prepared in Mueller-Hinton broth. Each well of a microtiter plate was filled with one hundred µL/mL of standardized bacterial suspension, ensuing in a remaining density of approximately 5×10^5 colony-forming units (CFU) / mL. The negative control was achieved through including one mL of phosphate-buffered saline (PBS) in place of grapefruit juice. The microtiter plate was then incubated at 37°C for 24 hours. The MIC was described as the lowest concentration of grapefruit juice at which no noticed growth change being seen.

Biofilm formation:

A complete quantity of 200 µL of sterile Luria Bertani supplemented with 0.1% glucose was placed in a well of the microtiter properly. Bacterial cells were inoculated into the wells at a density of 1×10^6 CFU / mL. Thereafter, all plates were incubated at 37°C for 24 hours to permit biofilm formation. Following incubation, the plates were softly washed thrice with sterile PBS. After washing, the biofilms have been fixed through including 200 µL of methanol in every well and left at room temperature for 15 mins. After fixation, the plates were re-washed thrice with PBS. In the long run, the biofilms were stained with 200 µL of 0.1% crystal violet solution and incubated at room temperature for 10 minutes. Gentle washing with distilled water was applied to the plates three times. Afterwards, all plates were air-dried at room

temperature. Later, the optical density (OD) was recorded at 600 nm using a microplate reader (BioTek, USA) [14].

Gene expression study; RNA extraction

The significantly affected *P. mirabilis* isolates were selected for investigating the impact of grapefruit juice on the gene expression of *mrpA*. RNA extraction from *P. mirabilis* biofilms has been conducted following the protocol supplied through SV Total RNA Isolation System (Promega, USA). A quantitative and qualitative assessment of the extracted RNA for next applications, a Quantus Fluorometer (Promega, United States) modified into applied. This evaluation entailed the application of 100 µl of diluted Quantifluor dye to one µl of extracted RNA. Following a 5-minute incubation period at room temperature, the RNA concentration was measured based on the obtained fluorescence information.

Primers

The primer sets applied in qPCR, as distinct in Table 1, were precisely designed for this work with the use of Geneious Prime 2023 software.

TABLE I: THE PRIMERS USED IN THIS STUDY.

Primer Name	Sequence (5`-3`)	Product size (bp)
mrpA-F	CACCCCAAGTGCT GCTCAAAA	130
mrpA-R	AGTCAGTGCGAAA GTTGCGAT	130
16SrRNA-F	ATGCAAGTCGAGC GGTAACAG	175
16SrRNA-R	TCCGATAGTGCAA GGTCCGA	175

qPCR

The assessment of gene expression for *mrpA* become accomplished, with normalization executed with the usage of 16SrRNA. The RT-qPCR mix becomes organized with the Luna general One-Step reaction mix (2X) at 10 µl, Luna WarmStart RT Enzyme mix (20X) at 1 µl, both primers (10 µM)) at 0.8 µl, and variable amounts of template RNA, adjusted to a complete extent of 20 µl with nuclease-free water. Furthermore, the thermocycling program was optimized through numerous trials. The final program comprised a reverse transcription step at 55°C for 10 minutes, and initial denaturation at 95°C for 60 seconds. Thereafter, denaturation was carried out at 95°C for 10 seconds for 40 cycles, followed by an extension step (60°C for 30 seconds). The steady MIC values determined across more than one isolate underscore the wide-spectrum interest of grapefruit juice towards the biofilm of *P. mirabilis*, highlighting its capability as a herbal alternative.

Statistical analysis

All experiments had been completed in triplicate. Kolmogorov-Smirnov and Shapiro-Wilk tests have been done to test the normality distribution of observations. Data (nonparametric variables) were expressed as median (range). Wilcoxon Signed-Rank test changed into applied to assess the impact of grapefruit juice on biofilm. The variations were considered considerable while $P \leq 0.05$. These statistical analyses have been carried out using GraphPad Prism 9.5 software.

III. RESULTS AND DISCUSSION

Minimum inhibitory concentration of grapefruit juice

Table 2 presents the minimum inhibitory concentration (MIC) levels of grapefruit juice against numerous *P. mirabilis* isolates. The findings displayed that most isolates, embracing P1, P2, P3, P4, P7, P8, P11, and P12, had a consistent MIC of 50%. This indicated an exceedingly excessive susceptibility of these isolates to the antimicrobial efficacy of grapefruit juice. In assessment, isolates P5, P6, P9, P10, P13, and P15 revealed a barely decreased MIC of 25%, representing that these isolates were relatively extra sensitive to the antimicrobial effects of grapefruit juice.

TABLE II: MIC VALUES OF GRAPEFRUIT JUICE AGAINST PROTEUS MIRABILIS.

Isolate code	MIC (%)	Isolate code	MIC (%)
P1	50	P9	25
P2	50	P10	25
P3	50	P11	50
P4	50	P12	50
P5	25	P13	25
P6	25	P14	50
P7	50	P15	25
P8	50		

The determined variation in MIC values of the isolates under test ought to stem from inherent variations in their susceptibility profiles, prompted by factors including genetic makeup and virulence elements [15]. Comparing those results with preceding studies, the present findings align with research demonstrating the antimicrobial

efficacy of grapefruit juice in opposition to various bacterial pathogens. Hayati *et al.* [16] have reported similar MIC values of grapefruit juice against *Porphyromonas endodontalis* and *Porphyromonas gingivalis* (biofilm causing root canal contamination). Similarly, it aligned with those of Osungunna and Onawunmi [17] demonstrating the antimicrobial efficacy of grapefruit juice in opposition to urinary tract pathogens. Apparently, the MIC values recorded in the present work are in parallel with those reported in Iraqi research comparing the antibacterial efficacy of grapefruit juice against *P. mirabilis* [8, 9]. Those studies highlighted the potential of grapefruit juice as an alternative healing agent for combating multidrug-resistant strains of *P. mirabilis*, especially in the context of urinary tract infections.

Moreover, the evaluation of literature records provides demanding situations because of the diverse methodologies employed in evaluating antibacterial efficacy, variations in solvent utilization, and disparities in the source and purity of tested materials, often stemming from distinctive plant extracts. The antimicrobial characteristics of natural compounds were elucidated now not only via the determination of MIC and minimum bactericidal concentration (MBC); but additionally through strategies along with agar well or disc-diffusion assays [7].

Biofilm

The findings of the present investigation revealed a significant reduction (P< 0.05) in the intensity of *P. mirabilis* biofilms following treatment with grapefruit juice, as evidenced by the aid of the records offered (Figure 1). Prior to treatment with grapefruit juice, the median biofilm thickness became 0.361 (0.218), whilst after treatment, the median thickness decreased to 0.245 (0.322).

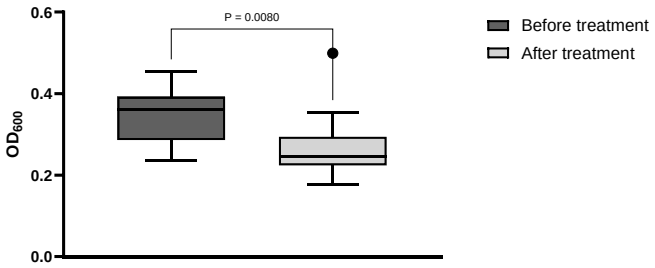

Fig 1: Effect of grapefruit juice on the Proteus mirabilis biofilm. Wilcoxon Signed Rank test. Z = -2.5, n= 15, P value = 0.008.

This vast reduction in biofilm intensity highlights the capability of grapefruit juice as a powerful agent against *P. mirabilis* biofilms. Preceding research has suggested the antimicrobial properties of grapefruit juice, which can be attributed to its diverse bioactive compounds, including flavonoids, phenolic acids, and organic acids found in grapefruit those compounds have been proven to inhibit the boom of numerous bacterial species [7, 18, 19]. Moreover, the findings of this observation are constant with the developing body of evidence helping the antimicrobial efficacy of natural compounds, along with fruit juices, as opportunity or adjunct treatment plans for bacterial infections. Fruit juices offer a promising horizon for addressing antibiotic-resistant pathogens and reducing the selective strain using antibiotic resistance [7].

Gene expression

The supplied data in Table 3 gives the fold change values for 4 exclusive isolates denoted as P10, P12, P13, and P14. Analysing the data, marked variations in fold change were found. P12 exhibits the highest value of 259.57, indicating an extensive upregulation. This shows that the issue upon discussion (which may be a gene, protein, or different biomolecule) is considerably overexpressed in isolate P12, potentially implicating its involvement in specific mobile processes or responses.

Further, isolate P10 demonstrates a high fold change value of 59.71, signifying a marked upregulation. Even as not as achieved by P12, this still indicates superb upregulation of the aspect in isolate P10. Conversely, isolates P13 and P14 exhibit less fold change values of 12.64 and 9.44, respectively. Although those values imply an upregulation, they're comparatively lesser than what is detected in P10 and P12.

TABLE III: EFFECT OF GRAPEFRUIT JUICE ON THE GENE EXPRESSION OF MRPA.

Isolate code	Before treatment *mrpA*	After treatment *mrpA*	ΔΔCt	Fold change
P10	23.42	24.76	-5.9	59.714
P12	22.16	20.75	-8.02	259.57
P13	17.98	19.44	-3.66	12.640
P14	22.52	25.28	-3.24	9.4479

The discrepancy between the upregulation of biofilm-related genes and the observed inhibition of biofilm formation while dealing with grapefruit juice may be attributed to multiple factors. first of all, grapefruit juice

incorporates antimicrobial compounds which include polyphenols and flavonoids [16], that may disrupt microbial increase and intrude with biofilm formation. Secondly, the complexity of biofilm formation entails numerous genes and environmental elements, making it prone to modulation with the aid of external factors like grapefruit juice [20]. Additionally, biofilm formation is a multifaceted system concerning numerous genes, proteins, and signalling pathways [1]. While the upregulation of genes associated with biofilm formation (such as *mrpA*) may additionally make contributions to this procedure, it's just one piece of the puzzle. Different factors, such as environmental situations and the presence of antimicrobial compounds like the ones discovered in grapefruit juice, can modulate biofilm formation in complex approaches. Universal, whilst grapefruit juice may also induce gene upregulation, its antimicrobial efficacy can override these consequences, inhibiting biofilm formation through; yet to discover numerous mechanisms.

IV. CONCLUSION

The steady MIC values determined across more than one isolate underscore the wide-spectrum interest of grapefruit juice towards the biofilm of *P. mirabilis*, highlighting its capability as an herbal alternative for fighting infections because of this opportunistic pathogen. Interestingly, the potential mechanism for this activity does not include the downregulation of the *mrpA* gene.

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