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Assessment of IL-6 and Evaluation of pharmaceutical compounds on biofilm-forming- *Acinetobacter baumanni* isolated from patients with urinary tract infection

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Abstract:

This study highlights the challenge of ineffective antibiotic treatment for urinary tract infections (UTIs) due to antimicrobial-resistant strains and biofilm formation by *Acinetobacter baumannii* (AB), particularly problematic in immunocompromised individuals. We aimed to investigate pharmaceutical compounds that could inhibit biofilm production in *A. baumannii* isolates associated with UTIs. In the study conducted from October 2023 to February 2024, interleukin IL-6 levels were measured using ELISA. Compounds Cinnamic(C) and Gallic (G) acids were evaluated for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) through broth microdilution. Bacterial susceptibility to antibiotics was assessed using the Kirby disk diffusion method and the Vitek-2 compact system with an AST card. Biofilm formation was analyzed using Congo red staining and a 96-well ELISA plate, and the efficacy of compounds C and G in treating biofilms was evaluated using the same method. Results showed that UTI patients had a mean IL-6 level of 19.00±1.581 pg/mL, significantly higher than the control group (mean IL-6 level: 7.400±1.140 pg/mL; $p < 0.0001$). Resistance rates among *A. baumannii* isolates were considerable, with varying percentages for different antibiotics. Gallic and cinnamic acids demonstrated antibacterial activity, inhibiting biofilm formation in *A. baumannii* at concentrations ranging from 0.5 to 128 mg/mL ($p \leq 0.01$). These compounds effectively suppressed biofilm formation across A. baumannii strains. In conclusion, IL-6 shows promise as a biomarker for diagnosing UTIs. Notably, gallic and cinnamic acids significantly reduced biofilms of extensively drug-resistant (PDR) *A. baumannii* strains, suggesting their potential therapeutic value against multidrug-resistant biofilms.

Keywords: *A. baumannii*; biofilm, multidrug-resistant, Interleukin-6

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Introduction:

Urinary tract infections (UTIs) are a significant public health issue, primarily caused by pathogens like Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis, and Staphylococcus saprophyticus[1,2]. Urinary tract infection (UTI) is a prevalent ailment, impacting an estimated 150 million people globally yearly. Approximately one-third of UTI patients experience complications in their recovery, with delirium being a common occurrence. Delirium is marked by symptoms indicating dysfunction in areas such as the frontal cortex and hippocampus. These symptoms include psychomotor agitation, lack of focus, and difficulties with short-term memory [3,4]. Despite the documented upregulation of systemic interleukin-6 (IL-6) and other inflammatory cytokines in systemic infection models, their role in the development of delirium remains unclear. A recent study using a mouse model of non-infectious acute lung injury showed that inhibiting systemic IL-6 reversed neuronal changes resembling delirium in the frontal cortex and hippocampus. This suggests that IL-6 may significantly mediate structural changes associated with delirium [5].

A. baumannii is a clinically significant pathogen, responsible for diverse nosocomial infections, notably affecting vulnerable populations such as ICU patients, Individuals in long-term care, those undergoing surgeries or procedures like central catheterization and tracheostomy, patients with enteral hemorrhage, and low birth weight neonates[6,7]. Acinetobacter baumannii holds a prominent position on the WHO priority pathogen list, labeled as "critical." This designation underscores the significance of this nosocomial pathogen, especially when it demonstrates resistance to carbapenem, considered a "last resort" antibiotic[8]. In recent times, antimicrobial resistance has emerged as a public health concern, posing a threat to communities and leading to a rising incidence of high morbidity[9] . The mortality rate from infections caused by MDR and XDR *A. baumannii* strains is high, with numerous outbreaks documented globally[10]. Biofilm development involves organized signaling with various genes and proteins regulating bacterial attachment to host cells. Biofilms form within 24 hours, following five main steps. After initial attachment, bacteria create a monolayer and produce a protective polymeric matrix. In the final stage, parts of the mature biofilm detach and disperse as planktonic cells, starting new biofilm formation elsewhere in the body[11,12].Given this dual challenge, several approaches are being considered to manage the spread of biofilm-forming multidrug-resistant *A. baumannii* strains [13]. Recently, there has been a growing interest in the use of polyphenols as a safe strategy for combating bacteria and biofilms [14]. Phenolic acids, including derivatives of “gallic and cinnamic acid”, induce irreversible alterations in bacterial plasma membrane characteristics, then in the leakage of vital intracellular components [15]. In terms of their ability to inhibit biofilms, phenolic acids are being investigated for quorum-sensing signal disruption, a process known as "quorum quenching." Quorum sensing plays a crucial role in biofilm-forming bacteria, making it a promising target for antibiofilm agents [14]. The current study aimed to determine IL-6 levels among UTI Iraqi Patients and inhibition of *A. baumannii-caused* UTI with phytochemical compounds.

Methods

Study Design

This research was carried out in the biotechnology department, College of Science, University of Anbar, Iraq, from October 2023 to February 2024. Five hundred mid-stream samples were collected under strict sterilization protocols before antibiotic treatment, ensuring sterility. Participants eligible were males aged 35 years or older.

Isolation of Bacteria

One hundred *A. baumannii* isolates were isolated from urinary tract infections (UTIs). These bacteria were cultured onto “MacConkey agar” and “blood

agar", following the instructions from Merck, Germany, and then incubated at 44°C for 24 hours.

Diagnosis of A. baumannii

Bacterial strain confirmation adhered to biochemical reaction protocols described in ref. [16]. Bacterial identification utilized both traditional methods, including culture media, biochemical tests (IMViC profile), and gram staining, as well as automated methods employing the automated Vitek-2 compact system (Biomerieux; France).

Detection of antibiotic resistance profile

The test profile was assessed according to CLSI guidelines using the VITEK 2 compact system. Additionally, the Kirby–Bauer disk diffusion method (Mast Group, Bootle, England) was employed for various antibiotics, including penicillins, cephalosporins, carbapenem, and Aminoglycosides.

Phytochemical Compounds

Phytochemical compounds were obtained from Thermofisher, a German-based company.

The dissolving of chemical compounds:

Stock solutions of "cinnamic" and "gallic acids" ("LOBA Chemie, Boisar, India") were initially prepared using previously described methods[17].For the cinnamic acid solution, 1.5 g of cinnamic acid was dissolved in 10 ml of dimethyl sulfoxide (DMSO) and then diluted with distilled water to 100 ml. The solution underwent sonication in a water bath for 2 hours at 80°C, with the gradual addition of 0.1 N NaOH until complete solubilization, followed by further dilution with distilled water to a final volume of 200 ml. For the gallic acid solution, 4 g of gallic acid was dissolved in 150 ml of distilled water and sonicated in a water bath for 30 minutes before additional dilution with distilled water to a final volume of 200 ml.

Estimation MICs of antimicrobial agents using the REMA method:

The Resazurin microtiter plate assay (REMA) determined the MIC of antibiotics and natural products. Sterilely, 100 µl of Mueller Hinton broth was added to each well of a 96-well plate, followed by twofold serial dilutions of cinnamic or gallic acid solutions. Then, 100 µl of a 0.5 McFarland standard overnight culture was added. Plates were incubated at 37°C for 18–24 hours, then 20 µl of resazurin was added, and incubation continued for 1-4 hours at 37°C[18]. MIC values were determined by visually identifying the lowest concentration at which the resazurin color remained unchanged, indicating no microbial growth.

Phenotypic detection of biofilm

Freeman et al. introduced the Congo red agar (CRA) method for qualitative detection of biofilm-producing microorganisms. This technique involves observing colony color changes on CRA medium, which contains 0.8 g Congo red, 36 g sucrose, and 37 g/L brain-heart infusion (BHI) agar, all from Merck, Germany. After incubating for 24 hours at 37°C, various colony colors enable distinguishing between biofilm producers (black, dry, crystalline) and non-biofilm producers (pink)[19].

Antibiofilm of cinnamic and gallic acids:

Firstly, 200 µl of bacterial suspensions at a 0.5 McFarland standard were dispensed into 96-well polystyrene microtiter plates. Then, 20 µl solutions of cinnamic or gallic acid at 1/2 and 1/4 of the MICs were added. The plates were then incubated for 24 hours at 37°C. After incubation, they underwent two washes with phosphate-buffered saline, followed by staining with 0.1% crystal violet. The dye was resolubilized using 33% acetic acid, and the optical density at 630 nm was measured using a microtiter plate reader (ELx800, Biotek). Each assay was conducted in triplicate, using wells without cinnamic and gallic acids as positive controls for biofilm formation [20]. Biofilm reduction percentage was calculated using the formula [(Ac - As) / Ac × 100], where "Ac" denotes the OD630 value of positive control wells and "As" denotes the OD630 value of

wells treated with cinnamic or gallic acids. Furthermore, the study investigated the anti-biofilm effects of sub-MIC levels of natural products, including cinnamic and gallic acids [21].

Exploring Synergistic Interactions of Gallic Acid, and Cinnamic Acid with Cephalosporin Antibiotics via Checkerboard Assay.

Two 96-well plates were utilized to evaluate the antimicrobial activity of two agents, *Gallic Acid, Cinamic Acid,* and an antibiotic. Serial dilutions were prepared horizontally for *Gallic Acid, and Cinnamic Acid* and vertically for the antibiotic. Each well-received Mueller-Hinton broth and the respective agents. *A. baumannii* was inoculated, and the plates were incubated at 37°C for 24 hours. The MIC, determined by resazurin stain, was used to assess synergistic effects via the fractional inhibitory concentration index (FICI). FICI values ≤ 0.5 indicated synergy, > 0.5-4 suggested an additive effect, and > 4 indicated antagonism. Synergy was noted when the combination's MIC was > 2 dilutions lower than that of the antimicrobial alone. Additive effects mirrored similar efficacy to individual agents, while antagonism resulted in a significantly reduced combined effect[22].

Assessment of IL-6:

Interleukin IL6 levels were measured using a human interleukin ELISA kit from SUNLONG Biotech Co., LTD (China), following the provided instructions.

Ethics of research:

"This research has been approved by a specialized research ethics committee with no.[2134] and date [9-6-2024] at Anbar University. The patient's verbal consent and signature were obtained.

Analysis of Research Data

Statistical analysis was performed using GraphPad Prism software (version 8.0). Chi-square and paired t-tests were utilized, with a significance threshold of $p < 0.05$.

Results:

Demographic criteria

Table 1 outlines the distribution of urinary tract infections (UTIs) and control groups based on gender. Among males, 24% (12 out of 50) had UTIs, while 56% (28 out of 50) comprised the control group. In contrast, among females, 76% (38 out of 50) had UTIs, with 44% (22 out of 50) in the control group. The results reveal a significant association between gender and UTI prevalence ($p = 0.0011^{**}$), with females exhibiting higher susceptibility. This aligns with existing literature citing anatomical differences, such as a shorter urethra, contributing to UTI prevalence in females. Gender disparity was also observed in the control group, potentially influenced by healthcare-seeking behavior or other health conditions. These findings emphasize the importance of considering gender variances in UTI prevention and management.

The assessment of Interleukin-6 (IL-6) levels among urinary tract infection (UTI) patients compared to the control group revealed significant differences. UTI patients exhibited a mean IL-6 level of 19.00±1.581 pg/mL, whereas the control group had a mean IL-6 level of 7.400±1.140 pg/mL. This dissimilarity was statistically significant with a p-value of <0.0001, as shown in figure1, and table2.

Table 1: Distribution of UTIs compared with control based on gender

Variables		Sample source				Sign.
		UTIs		Control		
		n	%	n	%	
Gender	**M**	12	24	28	56	**0.0011****
	F	38	76	22	44	
	T	50	100	50	100	

M: male; F: female; T: total.

Table 2: Assessment of IL-6 among UTI patients with control:

Name	M ± SD	P-value
Patients	19.00± 1.581	**<0.0001******
Control	7.400± 1.140	

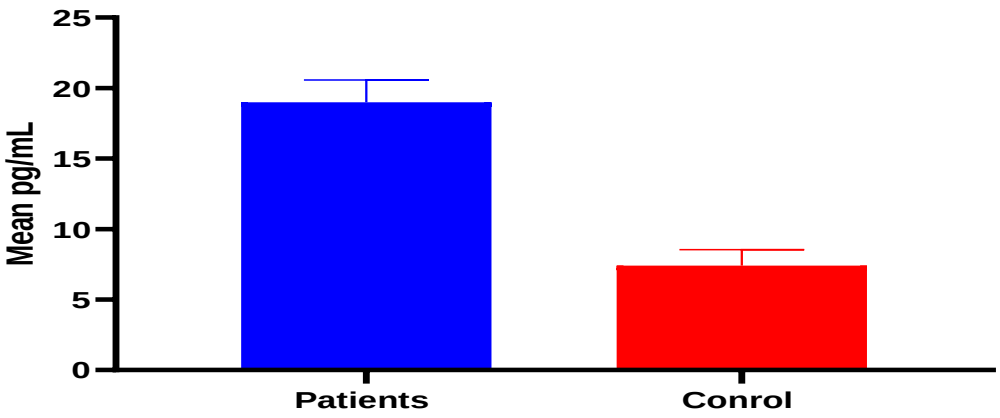

Figure 1 Comparison between patients and controls as regards urinary IL-6.

Diagnosis of A. baumannii

To confirm the diagnosis of *A. baumannii*, bacterial isolates were initially identified by growing them on "blood agar", and "MacConkey agar" under aerobic conditions.

Antibiotic resistance profile

According to CLSI interpretive criteria [23], the resistance rates among *A. baumannii* isolates to the tested antibiotics were as follows: Levofloxacin 35% (n = 35), Gentamicin 44% (n = 44), Imipenem 50% (n = 50), Cefipime 60% (n = 60), Ceftazidime 75% (n = 75), Ceftriaxone 85% (n = 85), Piperacillin-Tazobactam 95% (n = 95), and Ampicillin 100% (n = 100).

Biofilm formation in A. baumannii

The qualitative assessment of biofilm formation indicated that both *A. baumannii* strains exhibited black colonies on CRA with glucose. Out of the 30 tested isolates, 80% formed biofilms: 40% displayed strong biofilm production, 24% showed moderate, 16% weak, and 20% did not produce biofilms.

Estimation of MIC for antimicrobial agents against A. baumannii using REMA

The MIC values for the tested antibiotics and natural products ranged from 0.10 to 128 µg/mL. PDR-*A. baumannii* showed elevated MIC values for cefepime at 128 µg/mL. Conversely, SV extracts displayed a varied range of MIC values against the bacterial isolates, spanning from 0.10 to 0.21 mg/mL, as depicted in Table 3. In summary, the findings reveal synergistic interactions between specific combinations of antibiotics and natural products against *A. baumannii*, indicating potential therapeutic advantages in treating bacterial infections as shown in Table 3.

Table 4 and Figure 2 show that Pre-treatment, *Acinetobacter baumannii* showed a biofilm assay OD630nm reading of 0.1290±0.08486. Following treatment with gallic acid, there was a significant decrease in biofilm formation (0.03200±0.01947, p = 0.0002***). Similarly, cinnamic acid treatment led to a significant decrease in biofilm formation (0.02878±0.02270, p = 0.0004****). These results suggest both compounds effectively inhibit biofilm formation in *A. baumannii*, indicating their potential as adjunct therapies against multidrug-resistant infections. Further research is needed to understand their mechanisms and optimize treatment strategies.

Table 3: Synergism between phytochemical compounds and antibiotics against *A. baumannii*

Antibiotic	antibiotic MIC (mg/ml) By REMA	Phyto. Com. MIC	Combined Antimicrobials	FICA AN. N. P.	FICI (∑FIC)	Outcome
Cefipime	128	64	ceftriaxone + Cinnamic	0.25 0.10 mg/ml	0.35	**Synergism**
Cefepime	128	64	ceftriaxone + gallic	0.25 + 0.21 mg/ml	0.46	**Synergism**

Table 4: Treatment Biofilm forming- *A. baumannii with* ***gallic acid and cinnamic acid***

Name	A: Before Treatment (control) $M \pm SD$	B: After Treatment with gallic acid $M \pm SD$	*p*-Value	*B: After Treatment with cinnamic acid* $M \pm SD$	*p*-Value
Biofilm assay (at OD630nm)	0.1290± 0.08486	0.03200± 0.01947	0.0002***	0.02878±0.02270	0.0004****

M: Mean; SD: Std. Deviation; ***: strong significant.

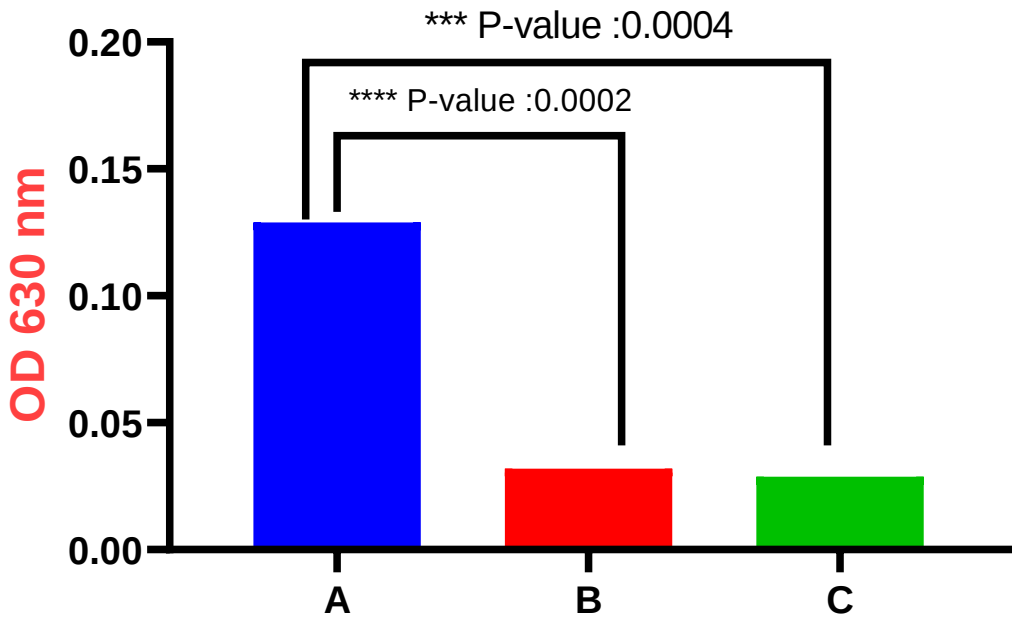

Figure 2: Inhibition of biofilm-forming- *A. baumannii* with pharmaceutical compounds. A: control (biofilm formation); B: **After Treatment with gallic acid; C:** ***After Treatment with cinnamic acid.***

Discussion:

This study highlights a significant link between urinary tract infections (UTIs) and elevated levels of Interleukin-6 (IL-6), a key pro-inflammatory cytokine in the immune response to infections and injuries. Elevated IL-6 levels commonly indicate the presence of inflammation or infection. In the context of UTIs, the increase in IL-6 levels is likely a response to the body's defense mechanism against invading pathogens, such as bacteria[24]. Sheu et al., [25] found that the urine level of IL-6 was significantly increased in patients with acute pyelonephritis than in lower UTI. Gurgoze et al. [26] observed a significant increase in serum IL-6 levels among children with acute pyelonephritis, demonstrating a sensitivity of 88% and specificity of 74%. Conversely, Mahyar et al. [27]conducted a study indicating that IL-6 and IL-8 have lower sensitivity and specificity compared to acute phase reactants like CRP. They concluded that these cytokines may not be dependable markers for distinguishing acute pyelonephritis from lower urinary tract infections.

The notable disparity in IL-6 levels between UTI patients and the control group emphasizes the potential of IL-6 as a biomarker for diagnosing and monitoring UTIs. Elevated IL-6 levels could facilitate early detection of UTIs, enabling timely intervention and treatment. Additionally, tracking IL-6 levels throughout UTI treatment may offer insights into treatment efficacy and infection resolution[28].

However, it's essential to recognize that while IL-6 proves valuable as a biomarker, its elevation is not exclusive to UTIs and can occur in various inflammatory conditions. Thus, clinical correlation with other diagnostic parameters is indispensable for accurate diagnosis and management[29].

Further investigation is necessary to explore the combined utility of IL-6 with other biomarkers or clinical indicators to enhance UTI diagnosis,

prognosis, and management. Moreover, researching IL-6-targeted therapies for UTI management could introduce innovative treatment approaches.

A significant concern arises from *A. baumannii* infections due to the increased incidence of multidrug resistance [30]. Compounding this issue is its capacity to develop biofilms [31]. The resistance of biofilms to antibiotics is approximately 1,000 times greater than that of planktonic cells, limiting the options for effective antimicrobial therapy[32].

Our isolates exhibited a substantially higher rate of biofilm production (100%) compared to recent studies of *A. baumannii* clinical isolates from Egypt, which reported a 70.1% frequency [33], Iran (70.6%) [34], and China (54%) [35]. Many studies have linked the high incidence of biofilm-forming - MDR *A. baumannii* with prolonged increases in resistance to strong stresses, such as dehydration and nutrient scarcity [36]. The relationship between resistance profiles and biofilm formation in *A. baumannii* remains somewhat controversial [37]. While some studies suggest a strong association between biofilm formation and multidrug-resistant (MDR) strains rather than susceptible ones [31], others have recently documented a relationship between strong biofilm-forming bacteria and antibiotic-resistant- bacteria [38].

Cinnamic acid has been studied as a potential alternative to traditional antibiotics against drug-resistant bacteria [39], [14]. Gallic acid has shown promise as an antibacterial agent, especially when combined with traditional antibiotics, but its effectiveness against drug-resistant *A. baumannii* is still limited [40], [41]. Cinnamic acid was more effective than gallic acid against bacteria, likely because cinnamic acid has fewer hydroxyl groups on its benzene ring [42], [14]

A notable finding of the present study was the effectiveness of both cinnamic and gallic acids in the inhibition of biofilm-forming - MDR *A. baumannii*. Previous research has shown that gallic acid can inhibit biofilm formation in various bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus mutans*[43]. Likewise, recent studies have acknowledged the antibiofilm effects of cinnamic acid derivatives [21]. There are different mechanisms to explain the activity of gallic and cinnamic acids against biofilm-forming -bacteria, including the breakdown of peptidoglycan within the cell wall, the inhibition of N-acyl homoserine lactones (AHLs)-mediated quorum sensing and antioxidant properties that prevent the formation of reactive oxygen species (ROS). As a result, these compounds may disrupt genic expression among biofilm-forming bacteria [44], [45], [43], [46].

Conclusion:

Interleukin-6 is considered a vital biomarker for diagnosing bacterial urinary tract infections, especially those caused by *A. baumannii*. This study illuminated the complex correlation between antibiotic resistance of *A. baumannii and* biofilm formation. The findings highlighted a notable connection between multidrug resistance (MDR) and the capacity for biofilm formation. Additionally, the research demonstrated that phytochemical compounds including gallic acid and cinnamic acid displayed significant inhibitory effects on biofilm formation in MDR *A. baumannii* isolates.

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