

Research Article

Antibacterial and antibiofilm activity of cinnamic acid against Multidrug-resistant bacteria isolated from Ramadi Teaching hospitals, Iraq

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Abstract

Various bacterial pathotypes remain a significant public health concern due to their pathogenicity and antimicrobial resistance. Moreover, the ability of bacteria to form biofilms can hinder host defense and antimicrobial eradication, leading to additional resistance. This study aimed to estimate the cinnamic acid's anti-biofilm activity against biofilms-producing bacteria. From October to November 2023, various clinical isolates were obtained from the Bacteriology Unit, Ramadi Teaching Hospital, Iraq. All isolates were identified using a conventional and automated VITEK-2 compact system. Based on the Clinical and Laboratory Standards Institute (CLSI) guidelines and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the Kirby-Bauer disk diffusion and AST were performed for antimicrobial susceptibility. Biofilm formation was estimated using a 96-well Enzyme-linked immunosorbent assay (ELISA) reader. Based on the antimicrobial susceptibility test, all bacterial isolates showed antibiotic resistance, including *Klebsella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. The data showed that MICs of cinnamic acid against bacteria were 125 µg/ml. The biofilms formed by all isolates exhibited strong strength (OD₅₇₀: 0.078-0.099). Cinnamic acid demonstrated significant inhibition of biofilm production in Multidrug resistance (MDR) bacteria (P-value = 0.0236). The results indicated that Cinnamic acid could be a promising anti-infective agent based on its ability to inhibit MDR-bacterial infections through biofilm formation.

Keywords: Antibiotic resistance, Biofilm, Cinnamic acid, Multidrug resistance

INTRODUCTION

Antibiotic resistance has emerged as a critical global health challenge, escalating both in community and hospital settings. The persistence and proliferation of multidrug-resistant (MDR) bacteria, and even pan-resistant strains that are impervious to all available therapeutic antibiotics, have resulted in treatment failures, leading to higher mortality and morbidity rates. Additionally, this situation considerably impacts the cost of medical treatments (Cepas *et al.*, 2019; Ahmed

and Al Meani, 2019; Abdulkareem *et al.*, 2021). Bacteria develop resistance to antibiotics through various changes and adaptations. These mechanisms include modifying the impermeability of the bacterial cell wall to prevent antibiotic entry, altering the drug's target site to reduce its effectiveness, and undergoing genetic changes like mutations in target genes and other mutational events. Additionally, bacteria can acquire resistance genes through small plasmids, mobile genetic elements that facilitate the transfer of resistance traits among bacterial populations (Qi *et al.*, 2016; Ahmed *et*

al., 2022).

Biofilms are characterized by clusters of bacteria adhering to a surface, and they play a vital role in the survival mechanisms of these microorganisms. Within a biofilm, bacteria are encased in a self-produced matrix, which consists of various substances such as polysaccharides (e.g., alginate), DNA, and proteins (Høiby 2017). Only recently, the significance of biofilm has been developed as a crucial microbial survival strategy, along with its substantial impact on various human activities, been recognized and understood (Claessen *et al.* 2014). The five stages of biofilm formation include i) Attachment, ii) Colonization, iii) Proliferation, iv) Maturation and v) Dispersion (Hussein *et al.* 2022).

In light of the increasing antibiotic resistance, there is a pressing need to search for alternative inhibitory drugs. Medicinal herbs, utilized for generations in treating infectious diseases, stand out as one of the most effective alternatives (Nikhat and Fazil 2022). Plants produce diverse secondary metabolites that protect them from various factors, including insects, microbes, herbivores, and other plants (Edan *et al.*, 2022). Numerous studies have highlighted that plant-derived chemicals exhibit additional biofunctions, making them potential candidates with antibacterial or antibiofilm properties, which is an intriguing approach. Various plants, such as *Aloe vera*, Chamomile (*Chamaemelum nobile*), and Garlic (*Allium sativum* L.), have been utilized for their therapeutic properties (Silva *et al.* 2016).

Contemporary advancements in science and technology are accelerating the discovery and development of new drugs that exhibit enhanced therapeutic activity while minimizing side effects. Plant chemicals are often employed in traditional medicine due to their perceived safety and long-established history of being used as immune stimulants and for preventing and treating various diseases (Ahmed *et al.*, 2023). However, the effectiveness of the resulting extracts is ultimately impacted by the extracted secondary metabolites, which depend on numerous extraction variables like time, temperature, and solvent (Ahmed *et al.*, 2023). Natural cinnamic acid has minimal toxicity to the majority of life forms. It is present in various plant species, primarily in *Cinnamomum zeylanicum*, where the word "cinnamic" originates (Franca *et al.*, 2021). In recent years, interest in CA and its derivatives has increased because of its many medicinal applications (Gunia & Krzyżak *et al.* 2018; Anwar *et al.* 2018). The present study aimed to know the activity of cinnamic acid against biofilm-producing bacteria isolated from Ramadi Teaching Hospitals, Iraq

MATERIALS AND METHODS

Study design

Between October and November 2022, a prospective

study was conducted at two hospitals in Al Ramadi city (General Ramadi Teaching Hospital, Ramadi Teaching Hospital for Women and Children) Iraq, focusing on multidrug-resistant (MDR) bacteria obtained from the bacteriology unit at Ramadi Teaching Hospital. The purpose of the ongoing research is to examine the impact of cinnamic acid on bacteria that are capable of producing biofilms. The study encompassed both Gram-positive and Gram-negative bacteria commonly associated with nosocomial infections. All bacterial isolates were isolated from clinically different sources, including wounds, sputum, urine, blood and burns. All isolates were streaked on MacConkey Agar, blood agar, and manitol salt agar that was prepared based on the manufacturer's instruction of Merck, Germany, and then incubated at 37 °C for 24 h.

Ethical approval

All participants obtained informed consent, which received approval from the ethical approval committee at Ramadi General Teaching Hospital in Al-Anbar Province, Iraq. A structured questionnaire was employed for data collection from both patients and controls, ensuring adherence to the guidelines established in the informed consent procedure.

Identification of bacterial isolates

Bacterial isolates, such as wounds, sputum, urine, blood, and burns, were obtained from various clinical sources. Isolated bacteria were identified using the biochemical identification cards of the Vitek® 2 Systems.

Antimicrobial susceptibility

The antimicrobial susceptibility test was conducted using the Kirby-Bauer disk diffusion method (Hudzicki 2009) for the bacteria indicated by the European Committee on Antimicrobial Susceptibility (GaCommittee *et al.* 2020). Various antibiotics used in the present work included Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefotaxime, Ceftazidime, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, and Trimethoprim/Sulfamethoxazole (Mast Group, Bootle, England).

Biofilm assay

The biofilm formation of bacteria was assessed following the method outlined by Ong *et al.* (2018). This was evaluated using a microtiter plate assay. First, an overnight culture of bacteria was diluted to a 0.5 McFarland standard. The bacterial culture was diluted to one-twentieth of its original concentration, and 200 µL aliquots were added to each of the three wells in a 96-well µL plate. The plates were incubated at 37°C for 24 hours. After incubation, the wells were washed with phosphate buffer saline (PBS) to remove any non-attached cells. The wells were then stained with crystal

violet, binding to the biofilm's proteins. The dye was then solubilized with acetic acid, which decolorizes the unbound dye. The bacterial culture was diluted 1:20, meaning 19 parts of sterile diluent were added to 1 part of the bacterial culture. This resulted in a diluted culture that was 1/20th the original concentration. 200 µL aliquots of the diluted culture were then added to each of the three wells in a 96-well microtiter plate. Negative controls, with no inoculum, were included to ensure that the absorbance readings were not due to background noise.

Acinetobacter baumannii ATCC19606 (HiMedia; India) is a strain of *A. baumannii* known to form biofilms. The study used it as a positive control to ensure that the experimental conditions were appropriate for biofilm formation. This strain is known to form biofilms, so its OD reading should be significantly higher than the negative controls. The OD cutoff value (OD_c) for biofilm formation was determined as three standard deviations above the mean absorbance of the negative control without the inoculum. Based on this criterion, the isolates were categorized as follows: i) Non-biofilm formers: OD ≤ OD_c, ii) Weak biofilm formers: OD_c < OD ≤ (2 × OD_c), iii) Moderate biofilm formers: 2 × OD_c < OD ≤ (4 × OD_c), iv) Strong biofilm formers: OD > (4 × OD_c).

Estimation of sub-minimum inhibitory concentration (Sub-MIC) for cinnamic acid against MDR-bacteria

In brief, 1% of the overnight grown culture of the test organism was added to 24 well microtitre plates (MTPs) containing LB broth supplemented with cinnamic acid ranging from 14 µg/ml to 1.5 mg/ml. The microtitre plates (MTP) were then incubated for 24 hours at 37°C. The sub-MICs (sub-minimum inhibitory concentrations) were determined as the highest concentration of cinnamic acid that did not affect the growth of the test bacteria, as indicated by the cell density (OD₆₀₀) measurement (Srinivasan, Santhakumari, and Ravi 2017). To evaluate the impact of the sub-MIC level of cinnamic acid on the growth of MDR-bacteria, 1 ml of the broth culture was collected at 2-hour intervals, and the optical density (OD) was measured at 600 nm over 24 hours to generate a growth curve. All subsequent anti-biofilm assays were conducted using the sub-MIC concentration (Banerjee *et al.* 2017).

Antibiofilm of cinnamic acid at sub-MICs

The biofilm formation was evaluated in various clinical samples of *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (pathogenic bacteria isolated from Ramadi Teaching hospitals) using a microtiter plate (MTP) assay. First, 200 µl of a 0.5 McFarland bacterial suspension was added to each well of a 96-well microtiter plate. After the bacterial culture was diluted and added to the wells,

20 µL of cinnamic acid solutions were added. The concentrations of the cinnamic acid solutions were half and one-quarter of these acids' minimum inhibitory concentrations (MICs). The plates were incubated for 24 hours at 37°C. After incubation, the plates were washed twice with phosphate-buffered saline to remove non-adherent cells, if any. The wells were then stained with a 0.1% v/v crystal violet solution.

After the dye was solubilized in 33% v/v acetic acid, the optical density (OD) of each well was measured at 630 nm using a microtiter plate reader (Tutar *et al.* 2016). Each assay was performed thrice, and wells containing no cinnamic or gallic acids were used as positive controls for biofilm formation. This means that these wells were expected to form biofilms, and their OD readings were compared to the OD readings of the wells containing cinnamic or gallic acids. The calculation of the biofilm reduction percentage was carried out using the following formula :

$$[(Ac - As) / Ac] * 100 \quad \dots\dots\dots \text{Eq. 1}$$

Where Ac = OD₆₃₀ value of the positive control wells;
As = OD₆₃₀ value of the wells treated with cinnamic acid (Shao *et al.* 2015).

Statistical analysis

All data was analyzed using graph pad prism, version 8.0. Chi-square and paired t-tests were employed, and statistical significance was defined as a p-value below 0.05.

RESULTS AND DISCUSSION

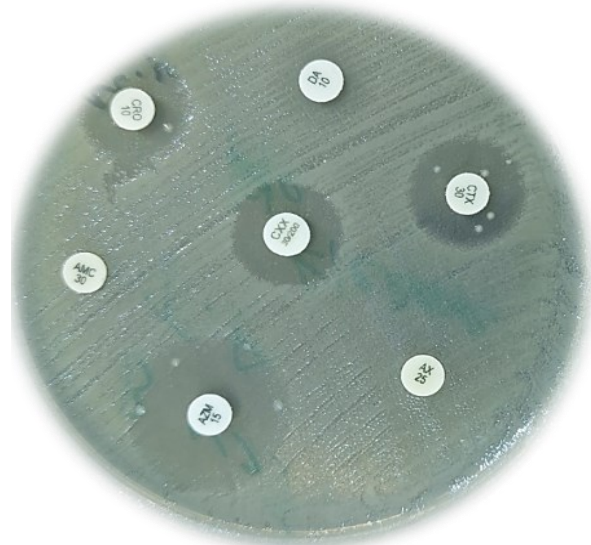
Antimicrobial susceptibility

Based on Table 1 and Fig. 1, the present study showed isolates of *S. aureus*, *E.coli* and *K. pneumoniae*, were resistant to all antibiotics, which included Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefotaxime, Ceftazidime, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Trimethoprim/Sulfamethoxazole were. While isolates of *P. aeruginosa* were resistant to all antibiotics except Piperacillin/Tazobactam. Almost all the studied isolates resisted various antibiotic families, except for the *P. aeruginosa* isolate, which showed sensitivity to the Piperacillin/Tazobactam disk. The isolates were categorized into susceptible, resistant (within 1-4 antibiotic categories), and multidrug-resistant (MDR) groups (resistant to three or more antibiotic families). In this study, all the isolates were identified as MDR since they exhibited resistance to three or more antibiotic families. For example, *K. pneumoniae* showed resistance to multiple antibiotic families, such as Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefotaxime, Ceftazidime, Cefepime, Imipenem, etc.

Table 1 also indicated that *P. aeruginosa* was resistant to more than one antibiotic family but remained sensi-

Table 1. Antibiogram parameter of different bacterial isolates according Kirbey-Bauer disk diffusion and AST card technique using Vitek-2 system; R : resistant ; I : intermediate ; S : sensitive

Antibiotics	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. aureus</i>
Amoxicillin/Clavulanic Acid	R	R	R	R
Piperacillin/Tazobactam	R	S	R	R
Cefotaxime	R	R	R	R
Ceftazidime	R	R	R	R
Cefepime	R	R	R	R
Imipenem	R	R	R	R
Meropenem	R	R	R	R
Amikacin	R	R	R	R
Gentamicin	R	R	R	R
Ciprofloxacin	R	R	R	R
Trimethoprim/Sulfamethoxazole	R	R	R	R

**Fig. 1.** Kirby-Bauer disk diffusion for bacteria . A: ceftriaxone ; B: Cefipime ; C: ceftazidime ; D: Amoxicillin/Clavulanic Acid ; E: Cefotaxime ; F: Gentamicin ; G: Amikacin

tive to the Piperacillin/Tazobactam family. Likewise, *E. coli* and *S. aureus* isolates resisted more than three antibiotic families, classifying them as MDR isolates.

Estimation of Minimum inhibitor concentration

The present data showed that MICs of cinnamic acid against bacteria was 125 µg /ml for all studied isolates, as shown in Table 2.

Biofilm assay

The study assessed biofilm formation in different clinical samples of *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* using a microtiter plate (MTP) assay. Four clinical isolates were screened for biofilm formation using the MTP method, and the results showed that all four isolates exhibited a biofilm-positive phenotype after 18 hours of incubation, indicating their ability to form biofilms. The strength of the biofilms was found to be strong for all isolates (OD570 : 0.078-0.099) (Fig. 2).

Antibiofilm of cinnamic acid

Based on Fig. 3 and Table 3, Cinnamic acid exhibited notable inhibition of biofilm production in multidrug resistant (MDR) bacteria, as indicated by a significant P-value of 0.0236

The escalating rates of multidrug resistance have given rise to significant concerns regarding bacterial infections. The situation is further exacerbated by the ability of these bacteria to form biofilms (Najeeb et al., 2022). The antibiotic resistance observed in biofilms is approximately 1,000 times higher than in planktonic cells, significantly limiting the range of available options for effective antimicrobial treatments (Hall and Mah 2017). All of these isolates (*K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *S. aureus*) under study were defined as multidrug resistance MDR because they were resistant to three or more antibiotics (Table1) . The emergence of resistance to commonly used antibiotics results from both patient misuse and inappropriate antibiotic use; several studies showed a significant correlation between biofilm production and multidrug resistance (Pramodhini et al., 2012). These results agree with Nirwati et al. (2019), who showed that almost all of the isolates of *K. pneumoniae* showed resistance to various antibiotics. The majority of these were also revealed to be biofilm producers. Another study on *P. aeruginosa* showed resistance to three or more antimicrobials (Roulová et al., 2022) . The study on *S. aureus* was isolated from burn and wound injuries and also showed MDR because it resisted 7 or 8 out of 11 antibiotics used (Alwash and Aburesha 2021) . Another study on *E.coli* showed multidrug resistance as resistance to at least three distinct antibiotics (Wu et al. 2021).

The present study found that *Acinetobacter baumannii* strains were resistant to aminoglycosides and β-lactams. Previous studies have also shown that amikacin resistance is common in imipenem-resistant *A. baumannii* strains, mainly due to the aminoglycoside resistance methyltransferase (ArmA) gene (Hasani et al. 2016); Upadhyay et al. 2018; (Bakour et al. 2014). Similarly, recent studies have found associations between

Table 2. Showing minimum inhibitor concentrations of cinnamic acid

Cinnamic acid	Serial dilutions								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
	µg	µg	µg	µg	µg	µg	µg	µg	µg
1000µg	500	250	125	62.5	31.25	15.625	7.8125	3.9062	1.95312

Table 3. Multidrug-resistant treated with cinnamic

Statistical	Biofilm formation	Treated with cinnamic	P-Value	P-value summary
Mean	1.301	0.09667	0.0236	*
Std. Deviation	0.1133	0.01550		
Std. Error of Mean	0.06542	0.008950		

*= significant (P < 0.05).

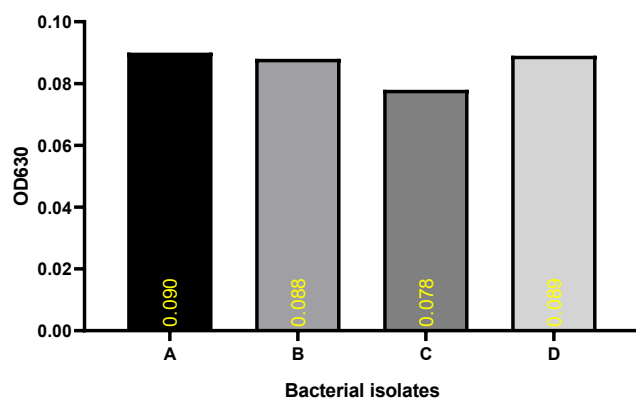


Fig. 2. Biofilm formation of bacterial isolates based on microtiter plate Elisa method; A : *KleibSELLA pneumoniae*; B.: *Staphylococcus aureus*; C.: *Pseudomonas aeruginosa*; D.: *Escherichia coli*

imipenem (IPM) and levofloxacin (LVX) resistance (Nour El-Din et al. 2021), which could be linked to various impacts arising from mutations at one or a few genetic loci (Adamus-Bialek et al. 2013). The isolates in the present study displayed a significantly higher frequency (100%) of biofilm production compared to other countries investigated: Egypt (70.1)(Asaad et al., 2021), Iran (70.6%)(Ranjbar and Farahani 2019), and China (54%) (Chen et al., 2020). Numerous studies have linked the elevated frequency of biofilm formation in MDR bacteria to extended survival and enhanced resistance to external stresses, including limited nutrients and dehydration (Badave and Kulkarni 2015); (El-Far et al. 2021); (Zhang et al., 2023).

The present study showed highly resistant-antibiotics and agreed with previous studies (Kyriakidis et al. 2021), (Bassetti et al. 2022), indicating a high prevalence of antibiotic resistance, which was employed in the present study's antibiotic resistance profiling, which revealed that the majority of the clinical isolates were resistant to all antibiotic disks used. Multidrug resistance in bacteria may occur because it assembles several genes, each of which codes for drug resistance. Resistance (R) plasmids are frequently the site of this resistance. Also changed target structure, enzymatic inactivation, increased expression of genes encoding for multidrug efflux pumps, etc., may also be

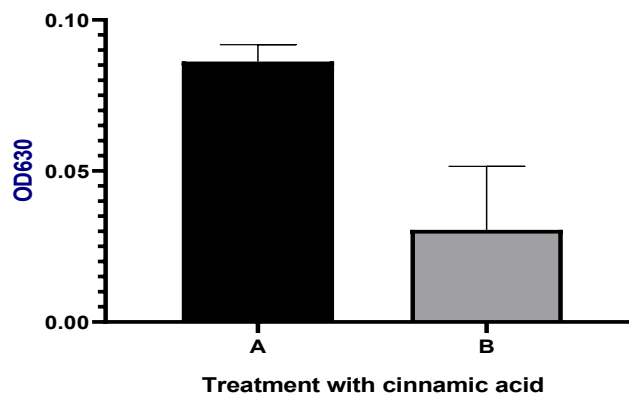


Fig. 3. Effect of cinnamic acid on biofilm forming – bacteria . A : Formation of biofilm ; B : treatment of biofilm with cinnamic acid

responsible for the MDR (Eliopoulos et al., 2008); Dey et al., 2022); Huang et al., 2022). On the other hand, the variation in antimicrobial resistance among different species depended on the sample type and its sources, such as urine, blood, or respiratory samples (Al Hamdan et al., 2022; Aldrazi et al., 2020).

Given the rise in antibiotic resistance, a quest for alternative inhibitory drugs is necessary. Medicinal plants have long been employed for treating infections; natural substances derived from plants, such as cinnamic acid, used for treating infectious diseases for ages, are one of the efficient alternative sources. Cinnamic acid's antimicrobial and anti-biofilm activity indicated the lowest MIC range (125mg/mL) against the tested isolates. Natural hydroxycinnamic acid, along with sinapic acid, ferulic acid, and caffeic acid, is a member of the class of chemicals known as phenolic compounds. These bioactive molecules have been identified as phenolic antioxidants (Kikuzaki et al., 2002). The analyses presented above revealed the magnitude of the issue posed by MDR to *A. baumannii*. Over the last decade, research efforts have focused on exploring natural remedies and products as alternative antimicrobial solutions to combat MDR isolates.

Cinnamic acid has been shown to have significant antimicrobial effects against many bacteria, including food-borne pathogens, standard isolates, and clinical iso-

lates(Guzman 2014). In the present study, the mean minimum effective dose (MED) of cinnamic acid against multidrug-resistant (MDR) bacteria was 125 µg/ml. This is significant as it suggests that cinnamic acid may effectively treat MDR infections. This finding is consistent with the previous study that has shown cinnamic acid to be effective against MDR bacteria (Sherif et al. 2021), which found that MICs of cinnamic acid ranged from 101 to 167 µg /ml. One interesting finding of the present study was that cinnamic acid effectively reduced the formation of biofilms associated with multidrug-resistant (MDR) bacteria. This is significant because biofilms can make it difficult to treat infections with antibiotics. Different phenolic compounds, like as derivatives of cinnamic acid, exhibit a range of mechanisms that contribute to their ability to inhibit biofilm forming bacteria. Many investigations have explored the antimicrobial, antibiofilm, and wound healing effects of cinnamic acid. These studies have shown that cinnamic acid possesses antimicrobial properties against Gram-positive bacteria and can disrupt biofilms formed by *Staphylococcus epidermidis*. Consequently, cinnamic acid shows promise as a safe treatment option for skin wound infections (Mingoia et al., 2022) . A new research study has shown that cinnamic acid effectively hindered the formation of biofilms in *K. pneumoniae* (Hussein et al., 2022).One suggested mechanism suggests that phenolic compounds may target the peptidoglycan within the bacterial cell wall, thereby influencing their ability to inhibit biofilm formation (Bali et al., 2019). Furthermore, another suggested mechanism potentially inhibits N-acyl homoserine lactones (AHLs)-mediated quorum sensing (Zhang et al. 2020). In the fact, these compounds demonstrate antioxidant properties, which can suppress the production of reactive oxygen species (ROS), consequently impeding the expression of key genes involved in regulating biofilm formation (Ong et al., 2018).

Conclusion

For first time, this research showed that cinnamic acid inhibited biofilm forming bacteria. Notably, Cinnamic acid (sub-MIC) demonstrates no adverse impact on the cell viability of MDR-bacteria, suggesting a reduced risk of developing drug-resistant strains. Moreover, the *in vitro* evidence further supports cinnamic acid's antagonistic activity against biofilm formation. Collectively, these findings highlight Cinnamic acid as a novel and promising anti-virulence agent for controlling infections caused by MDR bacteria.

Conflict of interest

The authors declare that they have is no conflict of interest.

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