

Interference with quorum sensing of *Klebsiella pneumoniae* by some plant extracts can affect the biofilm formation and antibiotic resistance

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Abstract

Quorum sensing (QS) is a perfectly orchestrated molecular communication system. It is a boon for *Klebsiella pneumoniae*, and bane for the host. This system is believed to make *K. pneumoniae* a leading cause of multidrug-resistant (MDR) nosocomial infections. This study aimed to investigate the antibacterial and anti-biofilm potential of medicinal plant extracts through interfering with QS of *K. pneumoniae*. The effect of different concentrations of ethanolic extracts of cinnamon and clove on *K. pneumoniae* was determined by analyzing the growth curve, survival assay (MTT), Qualitative and quantitative biofilm formation, antibiotic resistance, along with studying gene expression of the genes encoding the above traits, using quantitative real time polymerase chain reaction (qRT-PCR). The low concentrations of the plant extracts did not affect neither on the bacterial growth, nor on the viability of *K. pneumoniae*, supported with growth curves. Additionally, the biofilm production was inhibited even by lower plant concentrations, and both cinnamon and clove extracts were able to render the MDR bacteria to be more susceptible to antibiotics. At the molecular levels, the bacterium treated with either clove or cinnamon or in combination showed under expression of the biofilm formation regulation gene (*bssS*), the carbapenem resistance gene (*bla*), as well as the QS target gene (*LuxS*). Both clove and cinnamon ethanolic extracts exerted potent impacts on reducing pathogenic traits regulated by QS in *K. pneumoniae*. Finally, the study recommends further exploration of clove and cinnamon extracts separately or in combination to develop alternative therapies against MDR *K. pneumoniae* infections.

Keywords: Antibiotic resistance, Cinnamon, Clove, *Klebsiella pneumoniae*, Quorum sensing inhibitors.

Introduction

Multi-drug resistant (MDR) *Klebsiella pneumoniae* is a common cause of nosocomial infection, with a high morbidity rate ¹. This non-motile, encapsulated, great communicator Gram-negative rod has developed a powerful survival strategy, such as quorum sensing (QS) mechanisms, where cell-to-cell communication system regulates

the behavior of the bacterial colony, and the switching from individual cell strategy into whole colony acting as one cell ^{2,3}. Multi-drug resistant *K. pneumoniae* is considered the cause number one of lethal nosocomial infections in patients with burned skin in the major trauma centers in Iraq ^{4,5}. *Klebsiella pneumoniae* is well protected by its polysaccharides

capsule that shields the bacterium from the host immune provocation, including serum attack and cellular opsonophagocytosis; the capsule of the bacterium also triggers the inflammatory cascade in the host immune system, leading to septic shock. In addition, this microorganism possesses fimbriae to attach the bacterial cell to the host, and it sheds siderophores to collect iron ions from the host ⁶. A dense core genome including many bacterial virulence genes is another virulence component of *K. pneumoniae* ⁷.

Importantly, this bacterium produces extended-spectrum beta-lactamase (ESBL) that lyses the oxyimino cephalosporins to challenge the third-generation cephalosporins, including Penicillins, Monobactam, Aztreonam, and Cephalosporins ^{8,9}. Thus, Carbapenems are the main option for treatment in ESBL-*K. pneumoniae* infection ¹⁰. Yet, the Center of Disease Control (CDC) stated in 2013 that 80% of the reported Carbapenem-resistant cases were due to *K. pneumoniae* ¹¹.

Humans are the principal reservoir of *K. pneumoniae*; it is found in the guts of 5% to 38% of the general population and in the nasopharynx of up to 6% of the population ¹². Biofilm production is one of *K. pneumoniae*'s survival strategies for avoiding contact with the host's immune cells ¹³. Since 1882, when Friedlander first time isolated *K. pneumoniae* from a patient who died of pneumonia, the world has been in race against this powerful tool of death ¹⁴. It seems that the synthesized antibiotics are losing their magic and *K. pneumoniae* is still winning. Cinnamon and clove are common medical plant products, both

are traditionally used to treat skin, oral cavity, gastrointestinal and gynecological infections. Clove contains eugenol as a powerful antimicrobial agent, and the cinnamaldehyde in cinnamon prevents food spoilage due to foodborne microbes ¹⁵.

Al-fekaiki *et al.*, ¹⁶ demonstrated the antibacterial effects of the essential oil of *Cinnamomum zeylanicum* barks against *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas erogenous* and *Staphylococcus aureus*. This indicates the presence of broad-spectrum antibiotic compounds in that extract, which caused inhibition zone diameters ranged from 17 to 30 mm ¹⁶. Recent study recommended the use of clove and cinnamon oil as an effective treatment of infections caused by drug resistant (ESBL) *E. coli* and *K. pneumoniae* ¹⁷. Both *K. pneumoniae* and *E. coli* treated with eugenol-clove oil extracts showed significant cell membrane disruption, while cinnamaldehyde-cinnamon increased cell permeability causing cell lysis eventually ^{18,19}. These studies and many others, encouraged us to examine the safe usage of ethanolic extract of these important medical plants and to apply them as a potential alternative and effective medication. To our knowledge, no comprehensive study exists that covers many aspects of the interference of clove or cinnamon ethanolic extracts with *K. pneumoniae*, along with the molecular mechanisms of action of these plants. It is anticipated that the aqueous ethanolic extracts of these plants would be efficient in hampering the QS regulated traits of many microorganisms including *K. pneumoniae*. Thus, this study aimed to investigate the antibacterial and anti-biofilm potential of cinnamon and clove extracts by interfering with QS of MDR *K. pneumoniae*.

Materials and Methods

Bacterial Isolates

Highly virulent and MDR isolates of *K. pneumoniae*, clinically isolated from human burn-wound infections in the study of Sadeq and Lafta ²⁰, were used in this research.

Preparation of Plant Extracts

Two medicinal plant extracts were used in this study, including dried flower buds of clove (*Syzygium aromaticum* L) and bark of cinnamon (*Cinnamomum cassia* L). The herbs were purchased

from a local market in Baghdad city, Iraq, and they were identified and authenticated by Directorate of Seed Testing and Certification, Ministry of Agriculture, Baghdad, Iraq. Ethanolic plant extracts were prepared by maceration ²¹. Briefly, 50 g of the ground clove buds, and ground cinnamon bark were immersed in 500 ml of 70% ethanol/water. The mixture was incubated on a shaker incubator (Lab Companion, Korea) for 24 h at 37°C. The solution was filtered by filter paper (Whatman No.1) using

Buckner funnel under negative pressure, and the filtrate was incubated onto a sterile cabinet at room temperature in order to dry²². A stock of the extracts (1000 µg/ml) was prepared using sterile phosphate buffered saline (PBS, pH 7.4). The following concentrations were prepared: 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.625 µg/ml, 7.81 µg/ml, 3.9 µg/ml, 1.95 µg/ml, 0.98 µg/ml, and 0.49 µg/ml to be used in this study.

Growth Curves Analysis

Growth-curve analysis was performed for *K. pneumoniae* to determine the effect of the plant extracts on bacterial growth²³. The following concentrations of ethanolic extracts of cinnamon and clove were tested: 0.49 µg/ml, 7.81 µg/ml, 15.26 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml. Then, 100 µl of each concentration was added to suspension of *K. pneumoniae* healthy culture in nutrient broth (Tmmedia, India), counted 1×10^8 CFU/ml (equivalent to McFarland tube no. 0.5.). 96-well plates were incubated at the following intervals: 4, 6, 12, 24, and 48 h. Then, the optical density (OD) of the plates was read at 600 nm using microplate reader (Diareader, Austria). Three technical replicates were performed for each treatment, and negative (culture without extracts) and positive (absolute ethanol) control groups were included as well. Two biological repeats were also done for analyzing the growth curve.

The Cell Viability Test (MTT) Assay

NAD(P)H-dependent cellular oxidoreductase was used to evaluate bacterial cell viability²⁴. Briefly, 0.5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT) (Panreace, Spain) was used. A pure single colony of *K. pneumoniae* was incubated in Brain Heart Infusion (BHI; Tmmedia, India) broth for 24 h, at 37°C. Bacterial cells were collected by centrifugation (10,000 ×g for 3 min), washed twice with PBS, and suspended into a density of 1×10^8 CFU/ml in BHI. 100 µl of the bacterial growth was added to each well of the 96-well plate, mixed with 100 µl of cinnamon or clove extracts in the following concentrations:

62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml. The plates were incubated for 6, 12 and 24 h at 37°C, the no treatment-wells represented the negative control. The bacterial viability was tested by adding 10 µl of 0.5 mg/ml of MTT solution to each well of the 96-well plate. After incubation for 30 min at 37°C, Formazan crystals produced in the bacterial cells were dissolved into 100 µl/well of 1N of NaOH. The absorbance (OD) was measured at 550 nm by microplate reader. Four replicates were used for each concentration, with omitting the wells on the borders.

Biofilm Formation Test

Congo red agar (CRA) assay was performed to evaluate the biofilm formation ability of *K. pneumoniae*²⁵. The medium composed of 37 g/L BHI broth (Tmmedia, India), 50 g/L sucrose (Accusmix, India), 20 g/L agar powder (Promega, USA), and 0.8 gm/ L Congo red dye (GCC, UK). Four MDR *K. pneumoniae* isolates were streaked onto one quarter of the CRA agar medium and incubated aerobically at 37°C for 24 h. Black colonies with a dry crystalline consistency indicated biofilm production, while bad biofilm formers remained pink. Darkening of the colonies without dry crystalline colony appearance indicated an intermediate result.

Anti-Biofilm Activity Assay

The effect of the cinnamon and clove extracts on the biofilm formation by *K. pneumoniae* was evaluated using the crystal violet assay²⁶. These bacteria were grown on tryptic soya broth (TSB; Himedia, India) at 37°C in 96-well plate (flat-bottomed polystyrene plate). 200 µl of 1×10^8 CFU/ml was added to each well. Plants extracts of 100 µl were then added at the concentrations: 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml. Three 96-well plates were used for each treatment, biofilm formation was measured after 24 h of incubation at 37°C. The wells were gently washed three times with sterile PBS, to remove non-attached cells. The remaining biofilm was fixed by 99% methanol for 15 min. The plates were air dried and stained with 0.1% crystal violet (Accusmix, India), followed by washing with PBS. The biofilm formation was

quantified by reading at OD of 560 nm as well as the optical density of the control (OD_c) by microplate reader at the same absorbance. The biofilm production was estimated according to the following formulas: if $OD \leq OD_c$: then it is none biofilm producer, if $OD_c < OD \leq 2 \times OD_c$: then it is weak biofilm producer, if $2 \times OD_c < OD \leq 4 \times OD_c$: then it is moderate biofilm producer, and if $4 \times OD_c < OD$: then it is strong biofilm producer¹⁴.

Effects of the Plants Extracts on the Antibiotic Susceptibility

To estimate the activity of QS inhibitor plant extracts on reducing antibiotic resistance, a loopful of MDR-*K. pneumoniae* was streaked on BHI agar and incubated overnight at 37°C. The next day, the bacteria were harvested by PBS to make a suspension equivalent to McFarland tube no. 0.5 (1×10^8 CFU/ml). From the plant extracts, only 2 ml of the concentration of 125 µg/ml (either clove or cinnamon) were mixed with the same volume of the bacterial suspension in a sterile tube. Then, the mixture was incubated at 37°C for 16 h. Mueller Hinton agar (Himedia, India) was used to inoculate the mixture of the bacteria and the extract or the bacteria alone without treatment as a negative control. Afterward, by a sterile forceps, three different types of antibiotics (Liofilchem, Italy): Cefoxitin (Fox 30 µg), Penicillin G (P 10 IU), and Imipenem (IMI 10 µg) were placed and pressed slightly on the agar surface, along with PBS was added to a well created in the agar, as a negative control.

The Effects of the Plant Extracts on the Gene Expression

The effect of cinnamon and clove extracts on the expression of virulence genes of *K. pneumoniae* was tested using quantitative real time polymerase chain reaction (qRT-PCR). The QS target gene (*luxS*), biofilm formation regulation gene (*bssS*), and carbapenem resistance gene (*bla*) were tested, along

recA (encoding for recombinant DNA repair) as an internal control gene. The primers targeting the virulence genes were designed using Primer3web v4.1.0 online (<https://primer3.ut.ee/>). The bacterium grown overnight in BHI broth was treated separately with cinnamon or clove extracts (each 62.5 µg/ml) for 8 h, or the bacterial culture was treated with a mixture of 125 µg/ml of clove and 500 µg/ml of cinnamon for 24 h. Then, RNA was extracted using TRIzol reagent (TRANS, China), and the cDNA was synthesized using iScript™ (BioRad, USA). The reverse transcription reaction was carried out by the conventional PCR using Thermal cycler (Cole-Parmer, UK). The cycling conditions included 25°C/10 min, 42°C/15 min, and finally 85°C/5 sec. The qRT-PCR reaction mixture was prepared based on the SYBR Green protocol (Applied Biosystems, UK). The mixture consisted of 5 µl of cDNA template (500 ng/µl), 1 µl of 10 pmol/µl of each primer (Table 1), 10 µl of Bright Green qPCR master mix (Applied Biosystems, UK), nuclease-free water up to 20 µl. The qRT-PCR cycling conditions were: 95°C for 2 min (initial denaturation), 40 cycles of 95°C for 15 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension), followed by melt curve analysis, which was done to check the presence of primer dimers and amplification of single product. The quality of cDNA products was tested using TapeStation system technology-Automated electrophoresis (Agilent Technologies, USA). Bioinformatics were done by MIQE® software, and the CT (the threshold) values were determined for the amplified cDNA samples in every reaction. The *recA* gene was used as a housekeeping gene to normalize the levels of cDNA and the relative gene expression. The fold change in gene expression between the treated and control samples was calculated based on the method described by Livak and Schmittgen²⁷, as below: Fold change in gene expression = $2^{-\Delta\Delta CT}$, $\Delta\Delta CT = \Delta CT_{\text{treated sample}} - \Delta CT_{\text{control sample}}$, $\Delta CT = CT_{\text{target}} - CT_{\text{reference}}$.

Table 1. The primer sequences used in this study.

Gene	Forward and Reverse primer sequences	Amplicon size (bp)	References
	5' -----3'		
<i>recA</i>	F-TTAAACAGGCCGAATTCCAG R-CCGCTTTCTCAATCAGCTTC	99	28
<i>luxS</i>	F- GTCTACCAGTGC GGGACTT R- AGTTCGTCGTTGCTGTTGA	104	This study
<i>bssS</i>	F- ACCGTTGATAGCTACGATGC R- GTCGTTAGCCATAGCGTCTG	104	This study
<i>bla</i>	F- ATGAACGCTTTCCCATGAT R- TAGTGGATCTTTCGCTCCAG	104	This study

Statistical Analysis

All experiments were conducted at least three independent times, and samples were done in triplicate (N=3), statistical analysis and *p*-value was

analyzed using one-way analysis of variance (ANOVA) by the use of the Standard Least Squares procedure of JMP Pro 16.0 software (SAS, Institute Inc., Cary, NC, USA).

Results and Discussion

Growth-Curve Analysis

Klebsiella pneumoniae strains are well known to be MDR, with a reputation for being a major cause of severe life-threatening nosocomial infections²⁹. It is a quorum-sensing bacterium, with high ability of forming and responding to certain molecules called autoinducers that regulate the bacterium growth within the medium and makes it a successful survivor and virulent pathogen³⁰. Unfortunately, the mass production and the intense usage of synthetic and semi-synthetic antibiotics induced the rise of public health global problems called the MDR bacteria, such as *K. pneumoniae*³¹. A global alarm was raised regarding the high and accelerated level of drug-resistant bacteria³².

Clove and cinnamon were used in a simple and raw practice, for hundreds of years, as well as in a

few modern studies as potential antimicrobial drugs^{15,33}. In this study, *K. pneumoniae* was found to be susceptible to treatment with clove and cinnamon extracts compared with synthetic antibiotics. Roughly the same trend of bacterial growth was seen for the culture of *K. pneumoniae* treated with the plant extracts at low concentrations starting from 31.25 µg/ml to 0.49 µg/ml, indicating that they had no or slight inhibitory activity on the bacterial growth. On the other hand, the higher concentrations, particularly 125 µg/ml to 500 µg/ml of both plants resulted in a steady decrease in bacterial numbers started from 6 h till 48 h of exposure (Fig. 1). Importantly, consistent results were obtained when the experiment of growth-curve analysis was repeated for three biological replicates, indicating the reliability of the findings.

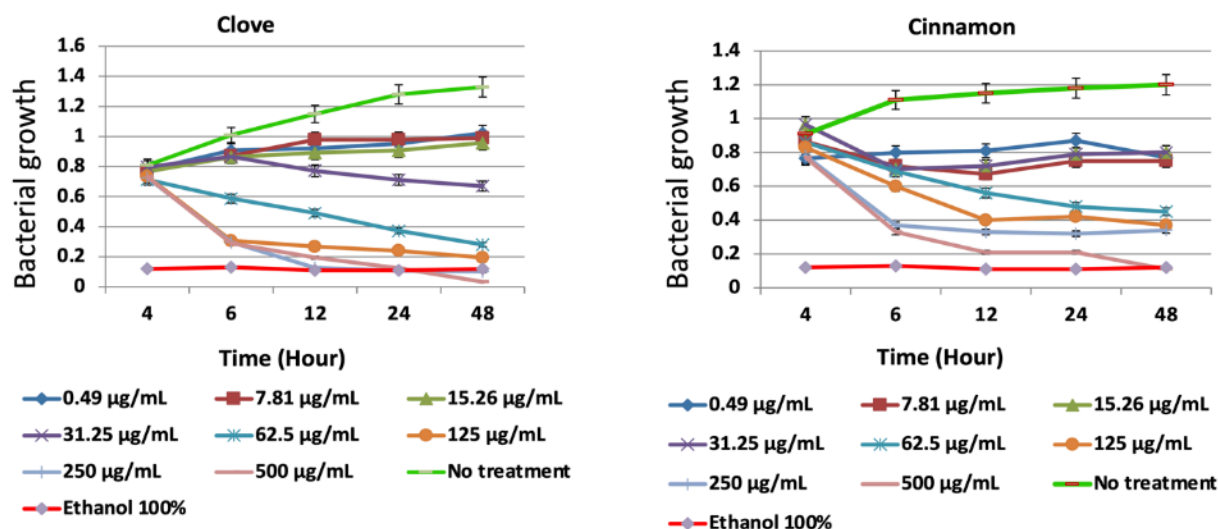


Figure 1. Effect of clove and cinnamon ethanolic extracts on the growth rate of *K. pneumoniae*. A growth curve analysis of non-treated and treated cultures was applied to specify MIC and MBC *in vitro* of the plant extracts. Different concentrations of clove and cinnamon were applied compared with negative control (no treatment) and 100% ethanol as a positive control. Readings were taken at different incubation times: 4, 6, 12, 24, and 48 h, N=3.

The effect of Clove and Cinnamon Ethanolic Extracts on *K. pneumoniae* Cell Viability

Fig. 2 shows comparisons of the survival rates measured by the MTT assay after 6, 12 and 24 h of treating *K. pneumoniae* with different concentrations of the ethanolic plant extracts compared to the non-treated bacteria. Adding 62.5 µg/ml of clove or cinnamon was able to keep the bacterial numbers at similar levels as the no-treatment control, and the relative survival was at stationary level up to 24 h of exposure (Fig. 2A, B). However, both bacterial survival fraction and cell viability started deteriorating after 6 h in the high concentration (500 µg/ml) of clove and cinnamon extracts (Fig. 2C, D). Similarly, significant decrease in viability was noticed when treating the bacterial cells with 250 or 125 µg/ml of clove extract for 24 h (Fig. 2C).

Interestingly, gradual reduction with time in the survival fraction was seen after treatment with either clove or cinnamon. While 20% decrease in cell viability noticed 6 h post treatment with clove, 40% reduction occurred after 12 h, and 60% reduced

viability happened after 24 h, supporting Anandhi *et al.*,³³ results that showed a significant reduction in bacterial growth after treatment with clove and cinnamon spray in different concentrations. Approximately similar scenario was seen in case of cinnamon exposure. Consistent findings were observed when this experiment was repeated, indicating the reliability of the data.

The findings of this study suggest that clove has more anti-bacterial efficiency than cinnamon, which is usually used as a part of combination not solo (Fig. 2). However, Mandal *et al.*,³⁴ revealed encouraging results where cinnamon had a successful effect on Methicillin-resistant bacteria. Another study was done on clinical samples from Iraq-Baghdad and Egypt-Mansura, the study proved the inhibitory effect of ten medicinal plants extract combinations (including clove and cinnamon at concentrations between 100 to 50 µg/ml), on beta-lactamase activity including MDR-*K. pneumoniae*^{35,36}. Furthermore, *K. pneumoniae* quorum sensing was seen to be inhibited by plant extracts without cytotoxicity effect³⁷.

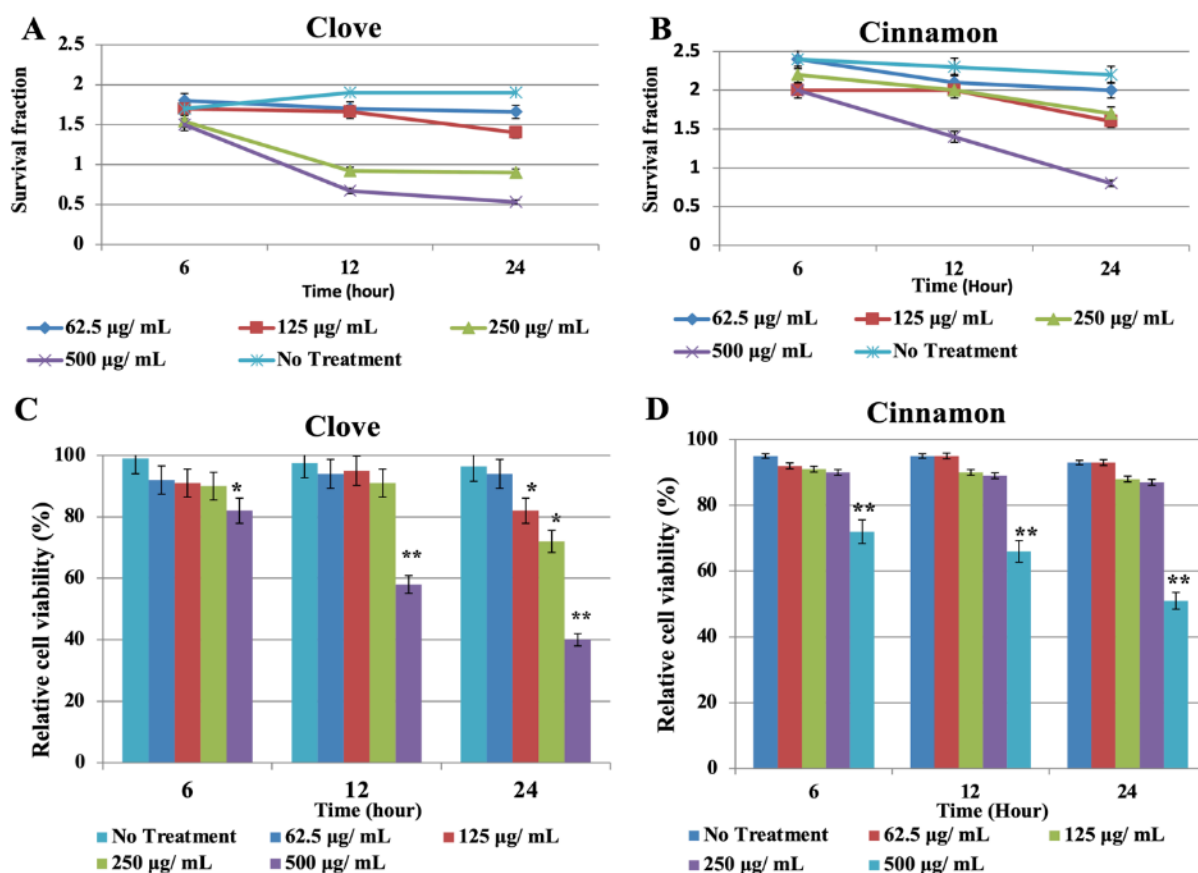


Figure 2. Survival fraction and relative cell viability of *K. pneumoniae* treated with different concentrations (62.5, 125, 250, and 500 µg/ml) of cinnamon or clove relative to no-treatment cultures after 6, 12 and 24 h of incubation. The results of OD (560 nm) were compared between treated and untreated cultures using One-way ANOVA test. The data are presented as means ± standard error and assumed to be statistically significant when $*=p<0.05$, $**=p<0.01$, $N=3$.

Detection of Biofilm Formation

The four multidrug-resistant *K. pneumoniae* isolates showed different capabilities to form biofilm on CRA medium (Fig. 3). Visual examination of the different isolates showed that among the four isolates only one was the strongest biofilm producer (A in Fig. 3) where clear blackening was observed compared to isolate C, which was non-biofilm producer.

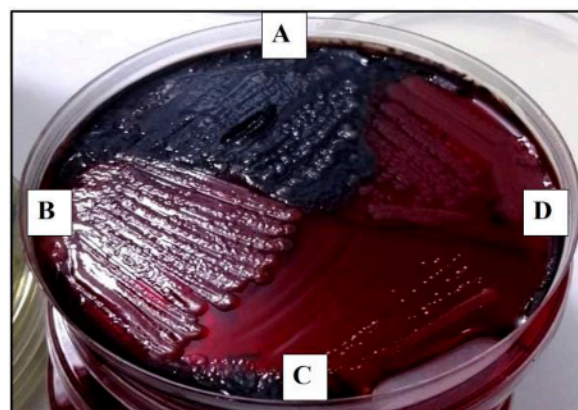


Figure 3. The four MDR *K. pneumoniae* isolates (A to D) grown on CRA plate. The isolates show variable degrees of biofilm production.

Quantitative Evaluation of Clove and Cinnamon Extracts on the Biofilm Formation

The crystal violet assay was successful in the quantitative analysis of the anti-biofilm potential of the plant extracts. Fig. 4 below illustrates that low concentrations of both plant extracts, particularly 31.25 µg/ml and 62.5 µg/ml, had a significant effect on the biofilm production ability, with slight interference with the bacterial growth. By contrast, clove and cinnamon extracts at higher concentrations (125, 250 and 500 µg/ml) had a significantly strong inhibitory impact on the biofilm formation after 24 h of incubation. Significantly, consistent findings were reported when this experiment was repeated for three biological replicates, indicating its reliability.

Thus, the plant extracts used in this study were able at sub-MIC to reduce the biofilm formation by *K. pneumoniae*. Elken *et al.*,³⁸ revealed vivid results

in using clove oil as an effective inhibitor of biofilm formation in Gram-negative bacteria. A strong correlation has been found between biofilm formation and pathogenicity of *K. pneumoniae*³⁹. The aforementioned study agrees with the current findings, it showed that various plant products including clove were able to stop biofilm formation and reduce the tolerance to treatment. Furthermore, the quantitative biofilm assessment (Fig. 4) of the current data is also supported by the findings of Higgas *et al.*,⁴⁰ and Adeosun *et al.*,⁴¹ particularly treatment with cinnamon and clove. Anandhi *et al.*,³³ and Vasconcelos *et al.*,⁴² studied the biochemistry of cinnamon and clove extracts, and found them to possess more than one type of active ingredient, including flavonoids as well as tannins and alkaloids, although they were at higher concentrations in clove than cinnamon.

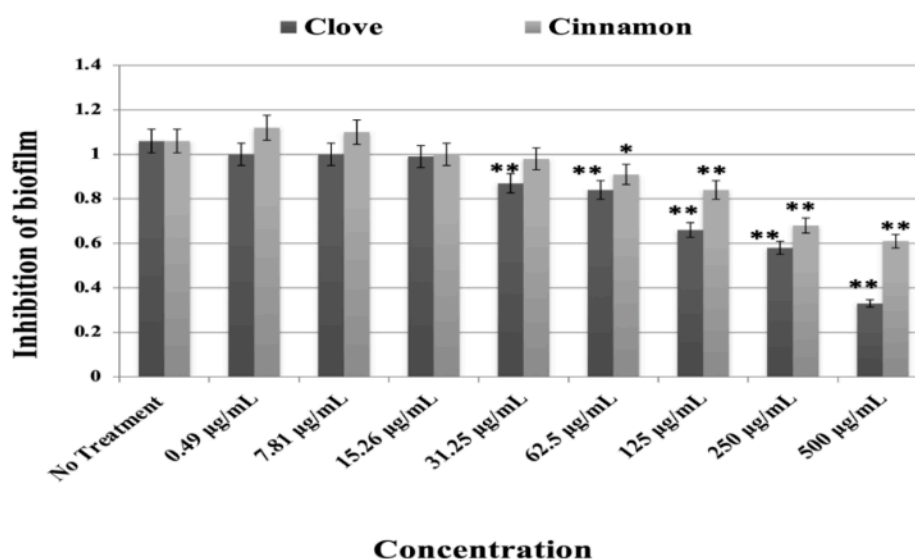


Figure 4. Inhibition of biofilm formation of *K. pneumoniae* by different concentrations of clove and cinnamon extracts using crystal violet method. The results represented by OD (560 nm) were compared between treated and untreated *K. pneumoniae* cultures using One-way ANOVA test. The data were presented as means \pm standard error and assumed to be statistically significant when $*=p<0.05$, $=p<0.01$, $N=3$.**

Effects of the Plants Extracts on the Antibiotic Susceptibility

Table 2 demonstrates that the MDR *K. pneumoniae* became susceptible to Penicillin G (10 IU) and Cefoxitin (30 µg) upon treatment with clove extract with diameters of zone of inhibition of 17 mm and 27 mm, respectively. Compared to the bacteria treated with cinnamon extract, an inhibition zone of

25 mm was reported for the Cefoxitin only. However, both extracts did not exert a significant consequence on resistance to Imipenem (10 µg). Similar results were obtained when this experiment was repeated several times, pointing to the reliability of the findings.

A previous study showed high efficacy of cinnamon oil extract combined with Streptomycin and

Ampicillin at low concentrations (MIC of 4.88 µg/ml)⁴². That study attributed the effective low MIC to the type of studied bacteria, or supposedly, the oil extract would be more stable than the

ethanolic extract, or the reason might be related to the concentration of the active ingredients in the extract.

Table 2. Inhibition zone diameters of antibiotics after treating *K. pneumoniae* with cinnamon or clove extracts.

Treatment	Antibiotic/ Zone of inhibition		
	Penicillin G (10 IU)	Cefoxitin (30 µg)	Imipenem (10 µg)
Bacteria alone	-	15 mm	-
Bacteria treated with clove extract	17 mm	27 mm	-
Bacteria treated with cinnamon extract	-	25 mm	-
PBS (-ve control)	-	-	-

Effects of Clove and Cinnamon Extracts on the Expression of Virulence Genes

The quality of cDNA products tested using TapeStation system technology showed clean and sharp band of PCR products compared to PCR products after 36 h of treatment, which revealed less prominent bands (data not shown). Fig. 5A demonstrates the qRT-PCR results of the bacterial culture treated with the plants combination, which caused clear suppression of the genes *LuxS*, *BssS*, and *bla* compared to no treatment control. The plants mixture also left-shifted the expression of the *LuxS* gene. Treatment of the bacterial culture with the

plants mixture resulted in cleavage of *LuxS* as shown by appearance of two bands of different molecular weights (100 and 150 kDa) in Fig. 5B. TapeStation system technology showed disruption in the expression of the three investigated genes; however, *bla* seemed to be the most affected by the mixture. A partial inhibition of gene expression was seen in the *BssS* and *LuxS* genes. Parallel effect of the plant extracts on inhibiting the expression of virulence genes was shown by Mirpour *et al.*,⁴³.

Liu *et al.*,⁴⁴ reported a significant reduction in carbapenem resistance of *K pneumoniae* after treatment with clove extract (Eugenol) in a dose dependent manner.

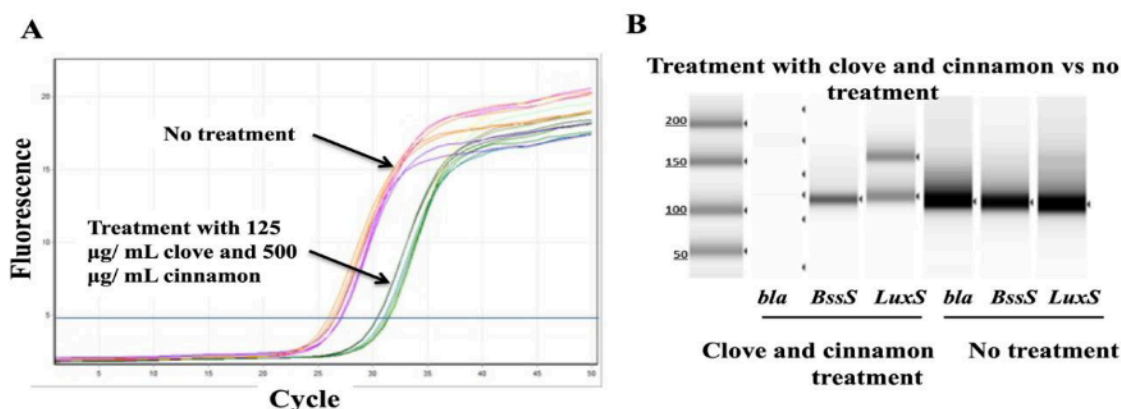


Figure 5. Virulence genes suppression by the mixture of clove and cinnamon; A: A plot of qRT-PCR cycle numbers against PCR products, and the curves represent CT values of *LuxS* (QS target gene), *BssS* (biofilm formation regulation gene), and *bla* (carbapenem resistance gene) in the cDNA of *K. pneumoniae* treated with a mixture of clove and cinnamon, or without treatment (PBS); B: The quality of cDNA products amplified by qRT-PCR and treated as mentioned before using TapeStation system technology.

Fig. 6 reveals the results of treating the bacterial culture with clove or cinnamon extracts separately. Fig. 6A shows clear suppression of all the tested genes, particularly *BssS* and *bla*, encoding the biofilm formation regulation and carbapenem resistance, respectively, in *K. pneumoniae* treated with the plant extracts separately. Compatible results were shown by Bahjat⁴⁵ in a clinical study performed on samples of pathogenic streptococci, where adding cinnamon and clove oil extracts prominently reduced biofilm formation. Sheng *et al.*,⁴⁶ showed that cinnamon oil inhibited the formation of Shiga toxin in *E. coli* on molecular basis. However, biofilm formation is a complicated multi-factorial process, as shown by another study focused on using clove oil to reduce formation of curli fimbriae in *E. coli*. In another study, Rajkowska *et al.*,⁴⁷ revealed the effect of clove oil anti-biofilm formation, and visualized the presence of clove oil on the cellular membrane of *Candida albicans*.

Pertaining to the QS target gene (*LuxS*), the plant extracts separately were also able to decrease its expression, clove was more effective than cinnamon (Fig. 6C). Decrease of gene expression of approximately 20% was noticed due to cinnamon treatment, whereas clove extract caused reduction of 40%. Due to the complicated nature of QS system and the involvement of more than one group of genes^{48,49}, further and more detailed studies are highly recommended. Topa *et al.*,⁵⁰ studied the effect of cinnamon purified active ingredient on QS system in *Pseudomonas aeruginosa*, their study unveiled some of the molecular bases of cinnamon anti-QS action. Kalia *et al.*,⁵¹ claimed that alcoholic and aqueous extract of cinnamon bark actively reduced the expression of *lasI/lasR* of QS-system in *P. aeruginosa*. The effect of cinnamon and clove on *K. pneumoniae* virulence is still not fully understood and more detailed studies are highly recommended.

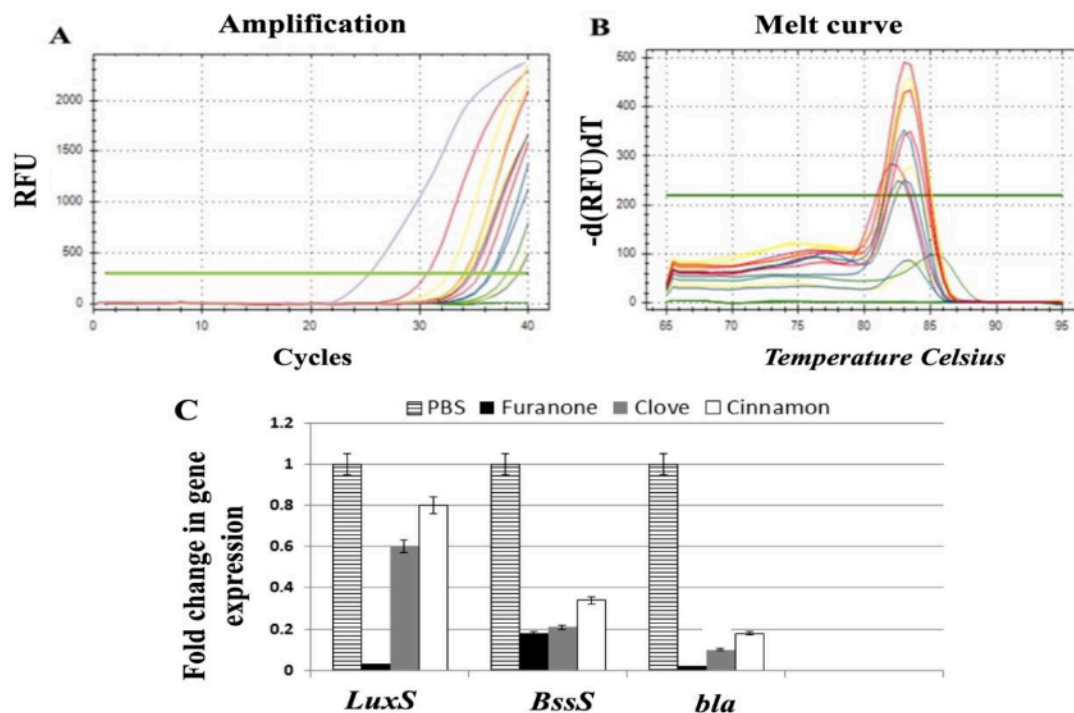


Figure 6. Suppression of virulence genes by clove and cinnamon. A: A plot of qRT-PCR cycle numbers against PCR products, and the curves represent CT values of *LuxS* (QS target gene), *BssS* (biofilm formation regulation gene), and *bla* (carbapenem resistance gene) in the cDNA of *K. pneumoniae* treated with either of the plant extracts or PBS (no treatment control); **B:** Melt curve analysis of the amplified genes shows no primer dimer with amplicons of specific targets; **C:** A representative bar graph reveals the quantitative analysis of qRT-PCR data by the fold change in gene expression of *K. pneumoniae* treated with clove (grey bars) or cinnamon (white bars) compared to the positive (furanone/black bars) and negative (PBS/ striped bars) controls.

Conclusion

To conclude, this study added valuable solid preliminary information to the field. Results highlighted the potential of clove and cinnamon ethanolic extracts to be used as an alternative or as treatment coordinates against *K. pneumoniae*. Clove and cinnamon extracts significantly reduced *K. pneumoniae* biofilm formation at sub-MIC levels, along with rendering the MDR isolate more susceptible to antibiotics already resistant to them.

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addition, many thanks are extended to the expert reviewers for their valuable comments.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, which are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No animal studies are present in the manuscript.

- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad, College of Veterinary Medicine, according to the Animal Utilization Protocol Certification, number 1078/ PG, which was issued on 16 May 2023.

Authors' Contribution Statement

ZES performed the experimental work, IJL conceptualization, designing the experiments, and writing and editing the final draft. SAL performed

some of the experiments and conducted the statistics. All of the authors participated in data analysis.

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التداخل مع استشعار النصاب لجرثومة *Klebsiella pneumoniae* بواسطة بعض المستخلصات النباتية يمكن أن يؤثر على تكوين الأغشية الحيوية ومقاومة المضادات الحيوية

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الخلاصة

استشعار النصاب (Quorum Sensing, QS) هو نظام اتصال جزيئي منسق بشكل مثالي، فهو نعمة للكليسيلا الرئوية، ولعنة على المضيف. يُعتقد أن هذا النظام يجعل هذه الجرثومة سبباً رئيسياً لعدوى المستشفيات المقاومة للأدوية المتعددة (MDR). هدفت هذه الدراسة إلى بحث القدرة المضادة للجراثيم والمضادة للأغشية الحيوية للمستخلصات النباتية الطبية من خلال التداخل مع QS للكليسيلا الرئوية. جرى تحديد تأثير تراكيز مختلفة من المستخلصات الكحولية للقرفة والقرنفل على هذه الجرثومة من خلال تحليل منحنى النمو، مقياس البقاء (MTT)، تكوين الأغشية الحيوية النوعية والكمية، مقاومة المضادات الحيوية، إلى جانب دراسة التعبير الجيني للجينات المشفرة لبعض عوامل الضراوة باستخدام تفاعل خميرة البلمرة المتسلسل الكمي في الوقت الحقيقي (qRT-PCR). لم يكن للتراكيز المنخفضة من المستخلصات النباتية أي تأثير، لا على نمو الجرثومة، ولا على حيويتها، مدعومة بمنحنيات النمو. فضلا عن ذلك، جرى تثبيط إنتاج الأغشية الحيوية عن طريق التراكيز النباتية المنخفضة، وكانت مستخلصات القرفة والقرنفل قادرة على جعل الجراثيم MDR أكثر حساسية للمضادات الحيوية. على المستوى الجزيئي، أظهرت الجراثيم المعالجة إما بالقرنفل أو القرفة أو كلاهما معا انخفاض التعبير الجيني لكل من الجين المسؤول عن تنظيم تكوين الأغشية الحيوية (*bssS*)، وجين مقاومة الكاربابينيم (*bla*)، وكذلك الجين المستهدف لـ QS (*LuxS*). كان للمستخلص الكحولي للقرنفل والقرفة تأثيرات قوية على تقليل الصفات المرضية التي ينظمها QS في الكليسيلا الرئوية. وأخيراً، توصي الدراسة بمزيد من الاستكشاف لمستخلصات القرنفل والقرفة بشكل منفصل أو مجتمعة لتطوير علاجات بديلة ضد الالتهابات الرئوية المقاومة للأدوية المتعددة.

الكلمات المفتاحية: مقاومة المضادات الحيوية، القرفة، القرنفل، الكليسيلا الرئوية، مثبطات استشعار النصاب.