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Inhibitory activity of clove oil and cinnamic acid on Klebsiella pneumonia biofilm

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ABSTRACT

New approaches must be developed to treat illnesses brought on by drug-resistant bacteria because of the rise in antibiotic-resistant bacteria and the insufficiency of new antibiotics on the market. Combining current antibiotics with phytochemicals (PCs) may be a method to boost antibiotic efficacy. Lab studies have linked essential oils (EOs) and their constituents to a set of phytochemicals that have these effects. As an illustration, we chose cinnamic acid (CAc) and clove oil (COs) as phytochemicals that can work in concert with antibiotics (cefepime) to combat an isolate of Klebsiella pneumonia. In the present study, initial evaluation of the biofilm forming abilities, haem agglutination, yeast agglutination & string test of 84 isolates of K. pneumoniae were performed. The strongest biofilm forming & positive virulence factors isolates was selected for further experimentation.

The COs and CAc's impact on early cell attachment was dosage dependent, according to the crystal violet (CV) assay (p less than 0.05). The inhibition of biofilm formation was highest at 4 MIC of clove oil then 0.25 MIC of CAc. When the CAc & COs were tested against a preformed biofilm, the percentage inhibition was increased with increasing with incubation time. As determined by the CV assay.

The methyl-thiazolyl blue tetrazolium bromide (MTT) assay results showed that the COs considerably decreased the metabolic activity of K. pneumonia biofilms at 4 MIC ($P< 0.05$). The addition of COS and CAC to prevent initial cell attachment lowered both the biomass of the cells (as shown by the CV assay) and their metabolic activity, resulting in 95.03% inhibitor of COs and 93.5% inhibitor of CAc. The K. pneumonia was grown on Foley balloon latex catheter (FBLC) and then calculated sessile cells. The number of cells shows statistically significant differences between adherent cells and free cells ($P<0.05$), and the median of growth for sessile cells treated with CAc & COs were reduced to almost half compared to untreated bacterial biofilm.

By using sonicating water bath (SWB), Foley catheter was put into SWB to dislodge the sessile cells in the solution to made suspension of bacteria, with measuring colony-forming unit (CFU), the suitable time was 6 min among (6, 10 & 15) min.

Synergistic interaction by combination between cefepime and phytochemicals (Cos & CAc), in cefipime-resistant strains of K. pneumonia, synergistic effects measured as metabolic activity decrease and restoration of cefipime sensitivity.

AMN3 “Human murine mammary adenocarcinoma” cell attachment phenotypes phenotypes appeared by K. pneumonia strains. The results showed the dissolution of the biofilm adhesions when treated with phytochemicals compared to the control.

Finally, based on K. pneumoniae's reported genome, genes (magA, Aerobactin, mrkJ, mrkA, AcrAB, blaKPC, blaCTX-M and entB) were found. To further confirm the inhibitory effects of the cinnamic acid and clove oil on the K. pneumoniae, which is strong biofilm-producing and their biofilm-associated genes (mrkA and mrkJ) were determined by quantitative RT- PCR. This study showed that the down-regulation of type III fimbria (mrkA) biosynthesis gene and Phosphodiesterase (mrkJ) responsible for biofilm development after treatment with cinnamic acid and clove oil.

Keywords: *Klebsiella pneumonia, MTT, FBLC, FIC, CFU*

INTRODUCTION

As a gram-negative bacillus, Klebsiella pneumoniae (K. pneumoniae) is a significant opportunistic pathogen that frequently causes nosocomial infections and contributes to substantial morbidity and mortality. As it is known to be associated with various infections such as Urinary Tract Infection (UTI) especially hospital-acquired Catheter-Associated Urinary Tract Infection (CAUTI), sepsis and pneumonia (1), (2)

Typically, bacteria bind to surfaces and form spatially structured communities inside a self-produced matrix, which consist of extracellular polymeric substances (EPS) known as biofilms (3), (4).

Biofilms provide defense and resistance to external stress, e.g., osmotic stress, desiccation, radiation, the host immune system, or harmful compounds, such as antibiotics. (5), (6). Due to their superior defense, pathogens arranged in biofilms are challenging to cure. They devise a variety of techniques to withstand high antibiotic doses, which ultimately results in persistent chronic infections. (7), (8). If the biofilm is broken up and the cells resume their planktonic state, the tolerance to antibiotics can be overcome, and the cells will once again be susceptible to them. (9). So, a different strategy for the management of biofilm infections could be the dispersal of existing biofilms in conjunction with antibiotic therapies. (10).

Inhibition via quorum sensing pathway interference and adhesion mechanism, disruption of extracellular DNA, exopolysaccharides, lipopolysaccharides, protein, and cyclic diguanylate messenger (c-di-GMP) involved in various signaling pathways are some of the different modes of action of anti-biofilm molecules addressed here.(11), (12)

The methods that are currently used for the control of biofilms include physical, chemical and more recently biological methods(13),(14).

Physical methods frequently utilized include heat plus mechanical scrubbing, brushing, scraping, and high-pressure spraying. (15) Chemical biocides including disinfectants, detergents, and preservatives are also frequently used. (16) On a smaller scale, the food business also employs bacteria and enzymes as biocontrol agents. (17),(18),(16). The enhanced resistance provided by sessile cells makes the majority of the control mechanisms for biofilm control, which are already in place, appear to be almost worthless. Plants have been considered as prospective candidates in the search for alternate biofilm control strategies because numerous herbal extracts have demonstrated antibacterial capabilities. (19)

Volatile oils, are frequently oily, unusually colored or transparent, complex, and their constituents are volatile, distinguished by a strong odor, and produced by aromatic plants throughout secondary metabolism. These substances exhibit a wide variety of pharmacological effects. (20). According to reports, volatile oils contain potent antioxidant, antibacterial, and antiviral properties. (21)

This study aims to use EOs to enhance the efficacy of antibiotics with a synergistic technique.

MATERIALS AND METHODS

Subjects

Between November 2020 and October 2021, the research was conducted over the course of a full calendar year. Al-Ramadi Teaching Hospital in Ramadi city-Iraq provided patient samples from 85 adult patients, 54 male and 31 female, ranging in age from 18 to 80.(22)

TABLE 1: Characteristics of the patients from whom K. pneumoniae was isolated

Characteristics		**n**	**%**
Gender	Male	54	63.53
	Female	31	36.47
Age (ys)	18-49	37	43.53
	50-79	48	56.47
Clinical Specimens	Sputum (Sp)	23	27.1
	Pus exudative (Pe)	17	20.0
	Urine (Ur)	15	17.7
	Wound swab (Ws)	12	14.1
	Catheters swab (Ct)	11	12.9
	Blood (Bl)	4	4.7
	Endotracheal tubes swab (Et)	3	3.5
Total		**85**	**100**

Bacterial Identification

From a clinical specimen, a collection of K. pneumoniae strains was collected. Afterwards, a quantitative culture was applied to MacConkey agar (Oxoid-UK) plate, and the plate was incubated at 37°C for 24-48 hours. Gram staining was used to identify the colonies, and the VITEK 2 system used biochemical identification to do so in accordance with manufacturer's instructions. A loopful of the BHI culture was injected into BHI-broth "Brain Heart Infusion-broth, Oxoid-UK" prior to each experiment, and it was then incubated for 24 hours at 37 oC. In order to conduct the experiments, an overnight culture that was diluted 100 times and matched to the 0.5 McFarland turbidity standard (about 10exp8 cfu/ml) was utilized.

Antibiotic Susceptibility Testing

In accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2020), the disc diffusion method was used to test antibiotic susceptibility (23). Amikacin, cefepime, cefotaxime, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam and trimethoprim/sulfamethoxazole were among the drugs included on the discs. According to CLSI 2020, the results were interpreted.

Biofilm Assay

Using a semi-quantitative method, the generation of biofilms by K. pneumoniae isolates was assessed. (24) In a nutshell, BHI-broth (Oxoid-UK) was used to prepare 0.5 McFarland suspensions of isolated K. pneumoniae, which were then inoculated into the wells of 96-well plates and incubated at temperature 37°C for 18 hr. using sterile BHI-broth, a negative control well was infected. Then, 200 µl of CV at a concentration of 0.5 percentage was used to stain the wells for 20 min, and the extra stain was removed using 200 µl sterile D.W. 3 times. After that, elution of the stain with 200 µl of 95 percentage ethanol (C2H5OH), and an enzyme-linked immunoassay reader assessed the optical density at λ570 nm. Triplicated standard deviations more than the mean optical density of the negative control (-ve) was considered to be the positive optical density (+ve). The strain of K. pneumoniae served as the positive control.

Cinnamic acid preparation

To obtain a 10 mg/ml concentration of cinnamic acid, 10 mg of cinnamic acid was dissolved in one milliliter of 10percentage Dimethyl sulfoxid (DMSO), mixed thoroughly using a vortex, and centrifuged at 8000 rpm for 10 minutes. The leftover was discarded, and the aqueous phase was taken and stored in vials at 4 °C until use.(25).

Extraction of clove oil

250g of fragrant Syzygium flower buds from the Myrtaceae family were used in this investigation. The powdered dry flower buds were added to a 1000mL flask. The essential oil was then obtained using hydrodistillation utilizing a glass (pyrex) Clevenger-type trap13 and 500 ml of distilled water. It was then put into amber vials with screw-top lids, dried with sodium sulfate (Na2 SO4) anhydrous, and kept in the refrigerator until it was needed.(26)

Agar well diffusion assay

Using the agar well diffusion method, cinnamic acid and clove oil's anti-bacterial activity was calculated. First, freshly produced Muller Hinton Agar (MHA, Oxoid, USA) was combined with 100 microliter of 24 hours bacterial (K. pneumoniae) cultures (in BHI) broth altered to a 0.5 McFarland standard during the cooling interval (42 oC) following autoclaving, and then poured into Petri dishes. Using a sterile cork borer, four wells (7 mm in diameter) were drilled into the plates after they had solidified. Test materials were placed in three wells and dissolved in 10% DMSO. As a negative control, the solvent solution (10% DMSO) was put into the fourth well. The zones of inhibition were determined after the plates had been incubated for 24 hours at 37°C (diameter in mm). (27), (28).

Assessment of minimum inhibitory concentration (MIC)

By modifying the Resazurin Microtitre-plate Assay (REMA), the minimum inhibitory concentration (MIC) of the antibiotic solution (cefepime), cinnamic acid, and clove oil (individually) were determined. Mueller Hindon Broth (MHB), 100 µl, was poured into each well of a microtitre plate under aseptic conditions. Following that, 100 µl of the test substance (1024 µg/ml cefepime, 256 µg/ml cinnamic acid, and 100% clove oil) were separately placed into the first row of the 96 well plates. Pipetting 100 l of the material test in successively decreasing concentrations of (½, ¼, ⅛, 1/16, 1/32,1/64 and 1/128) was used to perform the serial dilutions. Each well received 10 µl of a bacterial suspension containing 1.5 108 CFU/ml. They were incubated for 18–24 hours at 35–2°C while loosely wrapped in Parafilm. Following the initial incubation, Each well received 10 l of resazurin solution (Alamar blue), and the plate was once more incubated for an additional 18–24 hours, in order to see a color change. Resazurin's color changes were used to visually examine the data; shifts from purple to pink, red, or colorless were considered favorable alterations. The MIC value was determined as the lowest concentration at which resazurin's color did not change. (29), (30).

Synergism between phytochemical compounds and antibiotic

Checkerboard titration in 96-well microplates, in which the antibiotic was diluted along the columns and the antibiofilms were diluted along the rows of a microtiter tray, was used to evaluate the synergy between both the antibiofilm (cinnamic acid, clove oil) and antibiotic (cefepime).

The fractional inhibitor concentration (FIC) index, which is calculated as bellows, was used to express the results. FIC = ([A]/MICA) + ([B]/MICB), where MICA and MICB are the individual MICs of antibiotic A and antibiofilm B and [A] and [B] are the combined MICs of A and B. The following definitions were given for the FIC index: "(FIC ≤ 0.5), indicates synergistic action; (0.5 < FIC ≤ 1), additive influence; (1 < FIC ≤ 2), indifferent influence".(31)

Determination of biofilm inhibitory activity of cinnamic acid and cloves oil

Preventing early cell attachment On the first stage of biofilm formation, the impact of cinnamic acid and clove oil on cell attachment was assessed according to Sandasi team (32), with a few changes. Solutions of test materials (corresponding to "½, ¼, 1, 2, and 4" MIC) were produced in two separate flatbottomed 96 well microtitre plates. 100 µl of each solution were put to each well of sterile, dry 96-well microplates. Equal volumes of water were used as (–ve) controls, while ciprofloxacin (CIP 2 mg/ml) was utilized as a (+ve) control. The wells next received 100 µl of a 24 hours bacterial culture in BHI broth that had been modified to a 0.5 McFarland standard, resulting in a final measurement of 200 µl in each well (in triplicate). After being loosely parafilm-wrapped, to encourage cell attachment, the plates have been sterilized at 37°C for 24 hours. The development of biofilms was assess using the

crystal violet test (CV test) and the methyl-thiazolyl blue tetrazolium bromide (MTT) assay was used to measure metabolic activity.

Inhibition of preformed biofilm

According to Sandasi et al. (32), with certain modifications, the impact of cinnamic acid and clove volatile oil on biofilm maturity and formation was assessed. The development of biofilms was allowed to continue for 24 hours before the inclusion of the tested substances. In order to create biofilms, 100 µl of a 24-hour bacterial culture in BHI broth that had been adjusted to "0.5 McFarland standard" was transferred in triplicate into sterile, bottom 96-well microtiter plates' wells. The platters were lightly parafilm-wrapped and incubated at 37 degrees Celsius for twenty-four hours to promote cell adhesion and biofilm formation. Soon after incubation, 100 microlitter of each test substance (at a concentration that prevents cell attachment) to each well were added, resulting in a 200 µl required volume. Ciprofloxacin (CIP: 1 mg/ml) was used as a (+ve) control, and equal amounts of sterilize distal water (SDW) were added as negative (-ve) controls. The test ingredients were applied to prefabricated biofilms, and then the plates were incubated for 2, 4, 6, 12, and 24 hours. The biofilms were incubated, and the CV test was used to check for biomass adherence, and the produced biofilm cells were subjected to MTT assays.

Biofilm biomass assay

The modified crystal violet (MCV) assay was utilized to gauge the development of biofilms in K. pneumonia isolates, which was developed by Djordjevic and colleagues (33). The culture material of each well was removed carefully after the incubation time, and there were plates thoroughly cleaned by running them under sterile distilled water three to five times to remove any bacteria that had become loosely attached. The plates can air dry at room temperature and then be further baked at 60 to 65 oC for 45 to 50 minutes. The wells received 100 µl of 1% crystal violet, which was then left to sit for 15 minutes at room temperature. To get rid of the extra pigment, the wells were then cleansed 3 times with SDW. The wells were then destainted with 125 µl of 95 percentage of ethanol. A new plate was then filled with 100 µl of the destaining solution from each well, and the absorbance (abs) was calculated at λ595 nm utilizing a microplate reader. Based on the following equation, the mean absorbance (λ595 nm) was used to calculate the percentage inhibition of biomass development for every test material concentration: (19)

$$\text{Percentage inhibition} = \frac{\text{OD}_{595\ \text{Negative control}} - \text{OD}_{595\ \text{Experimental}} \text{X}100}{\text{OD}_{595\ \text{Negative control}}}$$

Biofilm metabolic activity assay

Evaluation of the biofilms' metabolic activities produced by K. pneumonia was estimate by MTT Blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] reduction, as described by Schillaci et al., (2008) (34). The MTT salt was dissolved in phosphate buffer saline (PBS) to yield a final concentration of 5mg/ml. After the cell attachment assay for 24h incubation and the preformed biofilm assay for 2, 4, 6, 12 and 24 h incubation, the plates were air-dried after the culture media had been gently removed. 100 microlitter of sterile PBS and 5 microlitter of a 5 mg/ml MTT solution were pipetted through every well, then incubated for a total of three hours at 37 oC. Further dissolving the opaque purple formazan in DMSO after being generated by the enzymatic hydrolysis of MTT. The absorbance at λ570 nm was then measured using the microplate reader.

Biofilm formation on Foley balloon latex catheter pieces

A sterile FBLC-Pieces (Foley balloon latex catheter pieces, ENTEPLIN size G16), which was cut within a biological safety hood, was used for each experiment 24h prior (to maintain sterile conditions). The FBLC-Pieces have been cut into (0.5 cm) length. In order to allow bio-film formation on FBLC-Pieces pieces, After overnight cultures were diluted (1/10exp4), 600 microliter from the broth culture were removed, this volume was placed into every well of a 48 well plate, with 1 piece of FBLC in each hole., then the plates were incubated at temperature 37°C for 24 h (freshly prepared). The FBLC-Pieces underwent an incubation period of "2, 4, 6, 8, 12 and 24" hours in BHIB (resent prepare) before being moved to new wells, at 37°C, and rinsed with SDW at RT. Unless otherwise stated,

every piece of FBLC was placed in 2 ml of BHIB after being incubated for 24 hours and then washed with sterile distilled water at room temperature.(35)

Dislodging sessile cells

Sessile cells were moved by sonicating a water bath (Grant, Ultrasonic Ltd). After washing with sterile distilled water, the pieces of FBLC or beads were placed underneath but not fully submerged in 2 cc of BHI broth in tubes falcon to prevent leakage and contamination. It was then vortexed for 20 s after being sonicated for (6, 10, and 15) minutes at 30,000 Hz in a WB-sonicator. Each microbial suspension were then properly diluted, placed onto the plate, and incubated at 37°C for an overnight period. CFU were measured using the Mils-Misra method after incubation, and data were reported as CFU/ml. (36)

Miles Misra method

The procedure was done according to Hedges (37) as follows: Inoculum/suspension was serially diluted through putting (1x) of suspension to 9x of diluent. At least 10−8 dilutions have been made. For each dilution series, three plates were needed, with an average of nearly three counts required for statistical reasons. The plate surface was dry enough to absorb a 20µl drop in 15–20 minutes. Plates have been split into equal areas (up to 6 can be used per plate). The dilutions labelled the areas. Suitable dilution of 1x 20 µl was dropped onto the media surface in each area, and the drop was permitted to spread normally. A drop from an elevation of 2.5 cm spread over a region of 1.5-2.0 cm in the original technique description. Prevent touching the agar layer with the pipette is necessary. The plates were left on the floor of the table to dry at 37oC for overnight before reversal and incubation. Every area has been noted for growth. High levels will result in concentrated growth over the fall region or big numbers of small/mixed colonies. Colonies are numbered in the area where it was possible to see the largest number of discrete full-size colonies (generally areas comprising between 2-30 colonies).

Cell line and culture medium

The Iraqi Center for Cancer and Medical Genetic Research (ICCMGR-Iraq-Baghdad) provided the AMN3 (murine mammary adenocarcinoma), which was maintained in RPMI 1640 (Sigma-Aldrich-Germany) coupled with 5% calf bovine serum (ICCMGR), 100 units/ml penicillin, and 100 µg/ml streptomycin.

Cell Adherence

The AMN3 cells were planted on 24well-PSP (polystyrene plates) for the adherence experiments, which were performed in duplicate four times. (38). By using Giemsa stain (GS) staining and light microscopy, 3 wells were employed to count the number of adhering bacteria, and to examine the adherence traits, one well was employed. In a nutshell, 10exp7 bacteria from overnight DMEM media, then cultured for “3 hours at 37°C” in a humid environment with 5 percentage carbondioxide. To count the adhering bacteria as colony-forming units, PBS was used twice to clean the wells at pH 7.4, treated with 0.1percentage of “Triton X-100”, serially diluted 10 times, and then plated on to NA-plates (CFUs). The mean data were stated as adherent CFUs, and these studies were carried out in triplicate over the course of three distinct days. A sample was stained with Giemsa, mounted on glass slides, and preserved with 300 mL of methanol. A “Nikon Eclipse microscope T 300-E” was used to view the stained samples.

Qualitative convential PCR

The following reaction mixtures were used in PCR experiments using a commercial PCR kit “Promega, Madison, WI 7.5 µl of Maser-Mix, 1.0 µl of primer F (10 µM), 0.5 µl of primer R (10 µM), 3.5 µl of water, and 2 µl of DNA for each template. PCR reactions were conducted in a thermal cycler Applied Biosystems by Thermo Fischer Scientific, Veriti 96 Well Thermal Cycler, USA”. PCR products were run in a 1.5 percentage agarose gel with TAE (Tris-Acetate-EDTA) at 150voit in an electrophoretic tank (Thermo Scientific, USA). Gels were stained with ethidium bromide (10 mg/ml) and visualized by UV light (MS major science, Taiwan).

Quantitative Real-Time PCR

Using "quantitative real-time PCR" (qRT-PCR), mrkA and mrkJ genes were assessed. qRT-PCR primers are mentioned in Table (5). In a nutshell, a "QuantiFluor® RNA System Kit (Promega, USA) was used to extract the total bacterial RNA. cDNA was then synthesized using an OneScript® Plus cDNA Synthesis Kit (abm®, Canada)".

Finally, "qRT-PCR was performed with a Luna® Universal qPCR & RT-qPCR Kit (New England Biolabs, USA) on the qPCR cycler (BIOER, China), with an initial incubation at temperature 95◦C for 1min, followed by 45 cycles of 15 s at 95◦C and 30 s at 60◦C". For all samples, the expression of mrkA and mrkJ was normalized using the reference gene (rpoD). The detection system software verified the threshold cycle (CT) values, and the data analysis were performed. using the "2−ΔΔCt method".

Statistical Analysis

The Statistical Package for Social Science (SPSS) version 24 was used to collect, review, code, and enter the data. Quantitative-data was provided as mean, ranges, and standard deviations, while the qualitative-data was displayed as amounts and percentages. To compare qualitative-data amongst each other, Chi-square was used. If the P-value was less than 0.05, it was deemed significant.

RESULTS AND DISCUSSION

The antibacterial effects of essential oils and each of their separate components have previously been studied. These hydrophobic substances' ability to break bacterial cell membranes by increasing cell permeability has been linked to their antibacterial activity. (39). 84 isolates of K. pneumoniae were used in the current investigation to perform preliminary assessment of the biofilm forming capabilities, haem agglutination (HAg), yeast agglutination (YAg), and string test. For additional testing, the isolates with the highest biofilm-forming capacity and the highest levels of virulence factors were chosen. Consequently, the current work shows how clove oil and cinnamic acid are effective at killing a particular strain of K. pneumoniae while also inhibiting the growth of biofilms.

Methanol and chloramphenicol served as the antibacterial positive and negative controls, respectively, as researchers employed the disk diffusion experiment to monitor the anti-bacterial activity of clove oil and cinnamic acid. A 34 mm "zone of inhibition" for cinnamic acid and a 28 mm zone for cinnamic acid were seen for the K. pneumoniae strain after an overnight incubation with the oil. The MIC of both clove oil & cinnamic acid was determined to be equivalent for the K. pneumoniae isolate (0.25% v/v & 2 mg/mL respectively). It has been noted that K. pneumoniae creates biofilms on abiotic surfaces found in hospital settings. (40)

Antimicrobials have been found to be more effective against biofilms than planktonic cells. There are various reasons for their resistance to antibiotics, including (i) extracellular glycocalyx, also known as glycocalyx, which acts as a physical barrier to prevent the diffusion of antimicrobials into the cells trapped in biofilms, is negatively charged, anionic, and present; (ii) Sessile cells express their genes differently than planktonic cells do; (iii) the deactivation of antimicrobial peptides; (iv) efflux pumps, which allow for the evacuation of these substances from the bacterial cell. (41), (42), (32)

In order to understand the anti-biofilm action of clove oil and cinnamic acid their effect were evaluated on both the initial cell adhesion by planktonic cells in addition on preformed biofilms (figure 1). The crystal violet (CV) assay indicated that the actually effect of clove oil and cinnamic acid on initial cell attachment was dosage dependent ($p < 0.05$). The inhibition of biofilm formation was highest at 4 MIC of clove oil then 0.25 MIC of cinnamic acid. These outcomes are explained by the fact that the creation of biofilms comprises two phases of attachment: a weak, reversible phase followed by a strong, irreversible phase. (43) The disruption of established biofilms that could be at the irreversible attachment phase therefore seems to require a greater concentration of clove oil. This highlights once more how biofilm-forming sessile cells are more resistant than planktonic cells. (44).

Overall, it appears to be a good strategy to deal with microbial adherence to condition the surface areas with natural substances to make them undesirable for attachment. (32)

When the cinnamic acid was tested against that a preformed biofilm, the percentage inhibition was increased with increasing with incubation time (Fig. 2). As assessed by the crystal violet assay.

The effect of clove oil was increased up to 6hr then reduced significantly with time (Fig. 2). The presence of a negative charge extracellular polysaccharide (EPS) layer in an actually performed biofilm, which may deter the entry of antimicrobials, or the compact 3D (three-dimensional) structure of a mature biofilm, which may obstruct the access of these compounds in to biofilm, can be responsible for the apparent resistance (R.) of the biofilm.(42) Another factor that may contribute to this increased resistance is the reality that the majority of antibacterial agents are more effective against cells that are actively growing. Due to their poor development rate and deficiency in nutrients and oxygen, the cells in biofilms may be less resistant to the antimicrobial actions of some substances. (32)

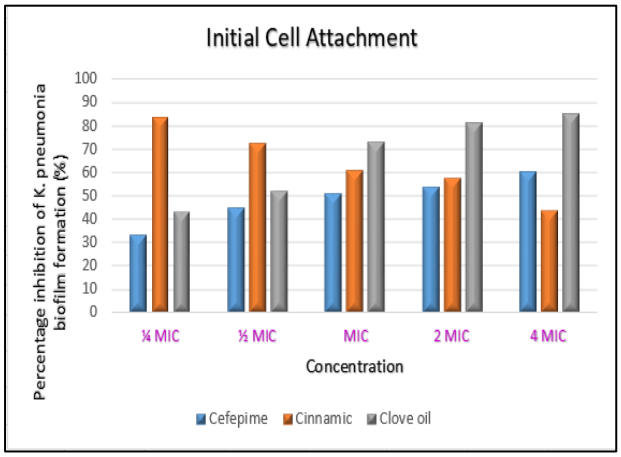

FIGURE 1. The effect of different antibiotic concentrations and phytochemical compounds on K. pneumonia's initial cell attachment is displayed as a percentage inhibition of biofilm development (%).

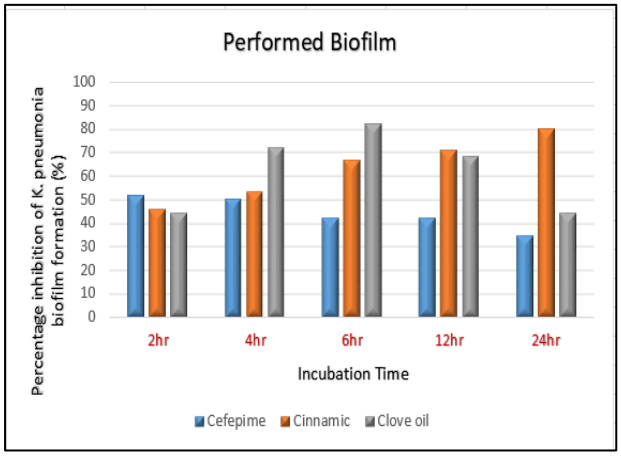

FIGURE 2. Percentage inhibition of K. pneumonia biofilm formation (%) on 24hours preformed biofilm of K. pneumonia.

The CV assay does not show the metabolic state of the cells, but it does serve as a biofilm's related biomass's indication. MTT is an azo dye salt that, when context in metabolically active cells, is decreased in to a calorimetrically detectable product, acting as a respiratory signal of live cells. (45) The MTT assay is used to determine if attached viable cells are present in biofilms,

whereas CV stains both potentially attached "viable and non-viable" cells. (46) The essential oil considerably decreased the metabolic action of the K. pneumonia biofilms generated at 4 MIC, according to the MTT assay results (p 0.05). Clove oil was added to prevent early cell attachment, and this decreased both the biomass of the cells (as shown by the CV assay) and their metabolic activity, resulting in 95.03 percentage inhibition of clove oil and 93.5 percentage inhibition of cinnamic acid.

However, it was discovered that the metabolic activity inhibition increased with greater exposure duration in the case of preformed biofilms, resulting decreased activity following a 24-hour exposure (Figure 2). The bulk of essential oils (EOs) are hydrophobic and attach to the lipid-rich cell walls, which explains why this is the case, increasing cell permeability and ultimately causing cell death. As a result, if the period of exposure is prolonged, so will the elimination of components from bacterial cell. (47)

Development of planktonic cells and biofilms on Foley Balloon latex pieces.

The aggregates formed primarily on the disposable Foley balloon latex catheter. Growth of K. pneumonia was analyzed under mobilization conditions. K. pneumonia was cultured onto FBLC. Viable counts was taken by sonicating FBLC and plating serial dilutions. Growth on the matrix was monitored for several periods. Under these conditions, K. pneumonia shows a growth pattern. The K. pneumonia was grown on FBLC and then calculated sessile cells. The number of cells shows statistically significant differences between adherent cells and free cells (P<0.05), and the median of growth for sessile cells treated with cinnamic acid, clove oil were (2.38E+04; 2.50E+03 respectively) and for planktonic cells (5.00E+06). With average and the median of growth for sessile cells treated with cinnamic acid, clove oil were (2.75E+05 ±8E05; 4.93E+04 ± 1E05 respectively) and for planktonic cells 1.26E+08 ± 2E508). See table 2.

TABLE 2: Progression of biofilms on FBLC-pieces at different times

Incubation times	Sonicating waterbath								
	5 min			**10 min**			**15 min**		
	Cinnamic Acid	**Clove Oil**	**Control**	**Cinnamic Acid**	**Clove Oil**	**Control**	**Cinnamic Acid**	**Clove Oil**	**Control**
2 hr	2.50E+04	2.00E+03	5.00E+05	2.75E+03	1.50E+03	5.00E+05	2.25E+03	1.00E+03	5.25E+05
4 hr	2.50E+04	2.25E+03	4.50E+06	2.25E+03	2.00E+03	4.50E+06	2.00E+03	2.00E+03	5.00E+06
6 hr	2.00E+04	2.75E+03	5.00E+06	2.00E+03	2.25E+03	5.25E+06	1.75E+03	2.00E+03	5.50E+06
8 hr	2.50E+05	2.00E+04	6.00E+05	2.50E+04	1.50E+04	6.00E+05	2.00E+04	1.00E+04	6.50E+05
12 hr	3.25E+05	3.00E+04	6.00E+07	2.75E+04	1.75E+04	6.25E+07	2.25E+04	2.00E+03	6.25E+07
24 hr	3.50E+06	3.50E+05	7.00E+08	4.00E+05	2.25E+05	7.00E+08	3.00E+05	2.00E+05	6.50E+08
Median	1.38E+05	1.14E+04	4.75E+06	1.39E+04	8.63E+03	4.88E+06	1.11E+04	2.00E+03	5.25E+06

Dislodging sessile cells using Sonicating water bath

By using Sonicating water bath, Foley catheter size sixteen was put into sonicating waterbath to dislodge the sessile cells in the solution to made suspension of bacteria, with measuring CFU by Mils Misra method, CFU was taken, the suitable time was 6 min (table 2). Our results correspond to Webber et al. (2015) (48). They concluded that the high-frequency cause inhibition of bacteria, because The cavitation effect, which occurs when cavities or bubbles form in a liquid media and produce some gas, may cause structural or functional alteration in the cells as a result of the breakage of molecular interactions.

The hydrodynamic feature of ultrasound destabilizes the biofilm structure at a specific frequency. The biofilm breaks down more quickly the longer it is exposed. When utilized at high frequencies, ultrasonic bath sonication can render bacteria inactive. Yet, when utilized at intermediate frequencies (between 20 kHz and 40 kHz), sonic cavitation acts to dislodge bacterial aggregation through its physical, chemical, and mechanical impacts.

Phytochemical Compounds and Antibiotic Solution Combination Assessment Using Checkerboard Method

Checkerboard assays of K. pneumonia gave synergistic effects when cefepime was combined with clove oil and cinnamic acid. The MIC values and checkerboard assay results of the cefepime, clove oil and cinnamic acid, (separately and in combination) for the clinical K. pneumonia select isolate are shown in table 3.

TABLE 3: Checkerboard assay results of the cefepime with (clove oil and cinnamic acid) separately

Test materials	MIC Value of A	MIC value of B	MIC value of (A) in combination with (B)	MIC value of (B) in combination with (A)	FICI value (ΣFIC)	Interpretation
Cefepime (A) Clove oil (B)	1024 µg/ml	0.195313 U/ml	2	0.024414	0.126952	Synergistic*
Cefepime (A) Cinnamic acid (B)	1024 µg/ml	2 µg/ml	4	0.5	0.253906	Synergistic*
* A synergistic impact between the investigated materials is indicated by FICI values below 0.5.						

The phenomena of antibiotic resistance can be effectively managed through the synergistic interaction of natural substances with the currently available antibiotics. Earlier research from this study has shown that combining cefepime with other substances has synergistic effects that are measured as a decrease in metabolic activity and a restoration of sensitivity to cefepime in K. pneumonia strains that resistant to cefepime. So Synergistic interactions of herbal therapy and PCs with antibiotics and other medicinally significant pharmaceuticals is a recent and efficient method for the management of resistant bacteria. Numerous compounds have been tested for their ability to modify resistance in order to combat microbial resistance; some of these compounds are successful against multiple targets, including penicillin binding protein (PBP), increasing the permeability of bacterial outer membranes and preventing bacteria efflux pumps.(49)

Study the cell adhesion using AMN3 cells before and after addition of phytochemical agents

Human murine mammary cancer cells were employed in this experiment to test for bacterial biofilm formation on the biotic surfaces. Also, we investigated the impact of clove oil, an anti-biofilm agent, on the development of bacterial biofilm, in the first three dilutions (0.1, 0.01, 0.001) as showing in table 4.

Our results showed a decrease by half in the number of CFU in the case of using clove oil as an antibiofilm. The cause is that antibiofilm affects bacteria's genetic material, preventing them from building biofilms or interfering with the pathways by which sugars are made, which are necessary for biofilm formation.(50), (51)

TABLE 4: Number of coloni forming unite (CFU) for treated & untreated AMN3 cells

Dilution	Control Untreated		Treated with Clove Oil	
	Cell count	CFU/well	Cell count	CFU/well
$1.0x10^{-1}$	271	2,710	119	1,190
$1.0x10^{-2}$	123	12,300	57	5,700
$1.0x10^{-3}$	62	62,000	16	16,000
$1.0x10^{-4}$	11	110,000	0	-
$1.0x10^{-5}$	0	-	0	-
$1.0x10^{-6}$	0	-	0	-

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$1.0x10^{-7}$	0	-	0	-
$1.0x10^{-8}$	0	-	0	-
$1.0x10^{-9}$	0	-	0	-
$1.0x10^{-10}$	0	-	0	-

Human murine mammary adenocarcinoma (AMN3) cell adherence phenotypes exhibited by K. pneumonia strains. The collection of K. pneumonia strains was identified 2 levels of adherence: A-AMN3 cells alone, B- control Untreated cells highly adherence (cells untreated with clove oil), and C- poorly adherence (cells treated with Clove Oil). Following Giemsa staining of the contaminated monolayers. As showing in the following micrographs were obtained and visualize using light microscopy "Nikon Eclipse microscope T 300-E" as showing in figure 3.

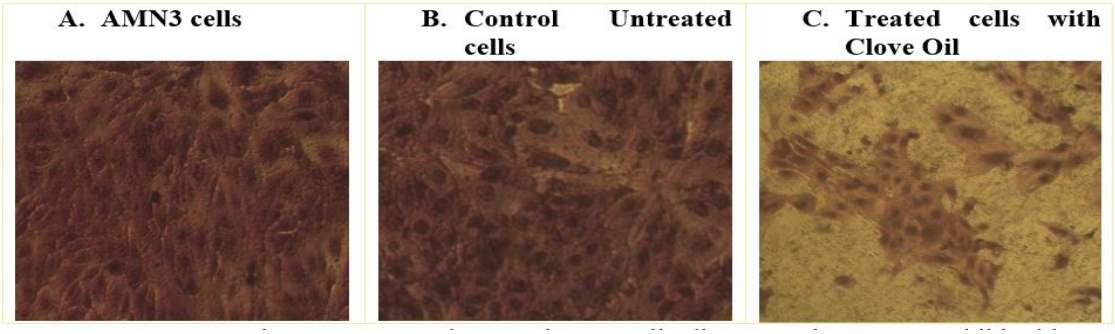

FIGURE 3. AMN3 cell attachment phenotypes exhibited by K. pneumonia.

Molecular Study of K. pneumoniae

Detection of virulence genes of K. pneumoniae

Based on the published Klebsiella pneumoniae genome, genes (magA, Aerobactin, mrkJ, mrkA, AcrAB, blaKPC, blaCTX-M and entB) were found. The Basic Local Alignment Search Tool (BLAST) tool was used to evaluate the nucleotide sequences acquired for each gene. Using the Primer-BLAST tool, primers were created for the sequence with the acceptable identity "score."

TABLE 5: The primers sequences for virulence genes of K. pneumoniae.

Gene	Primer Name Sequence (5'→3')		Product Size bp	Annealing temp.°C	Reference
magA (K1)	F	GGTGCTCTTTACATCATTGC	1282	53.4	(1)
	R	GCAATGGCCATTTGCGTTAG			
Aerobactin	F	GCATAGGCGGATACGAACAT	556	56.1	
	R	CACAGGGCAATTGCTTACCT			
mrkJ	F	TTTCGAGGTAACCGAAAACG	75	56.1	(52)
	R	GAGGTATCCTGTGGGCTCTG			
mrkA	F	TAAGCAAACTGGGCGTGAA	358	56.6	
	R	TAGCCCTGTTGTTTGCTGGT			
AcrAB	F	ATCAGCGGCCGGATTGGTAAA	312	63.6	(2)
	R	CGGGTTCGGGAAAATAGCGCG			
blaKPC	F	CGTCTAGTTCTGCTGTCTTG	797	54.6	
	R	CTTGTCATCCTTGTTAGGCG			
blaCTX-M	F	TGCGGTATTATCCCGTGTTG	216	59.2	
	R	TCGTCGTTTGGTATGGCTTC			
entB	F	CTGCTGGGAAAAGCGATTGTC	400	65.2	
	R	AAGGCGACTCAGGAGTGGCTT			
rpoD	F	TCCGGTGCATATGATTGAGA	105	87.5	(53)
	R	ATACGCTCAGCCAGCTCTTC			

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*** F: Forward sequences, R: Reverse sequences**

Gene expression by qPCR

The results of our study showed that K. pneumoniae species' most common virulence genes (shown in: table 6, and figure 4.) were blaCTX-M (93%), mrkJ (87%), AcrAB (73 %), entB (73%), and the lowest frequency gene were detected for Aerobactin (40%), mrkA (40%), blaKPC (20%), magA (7%).

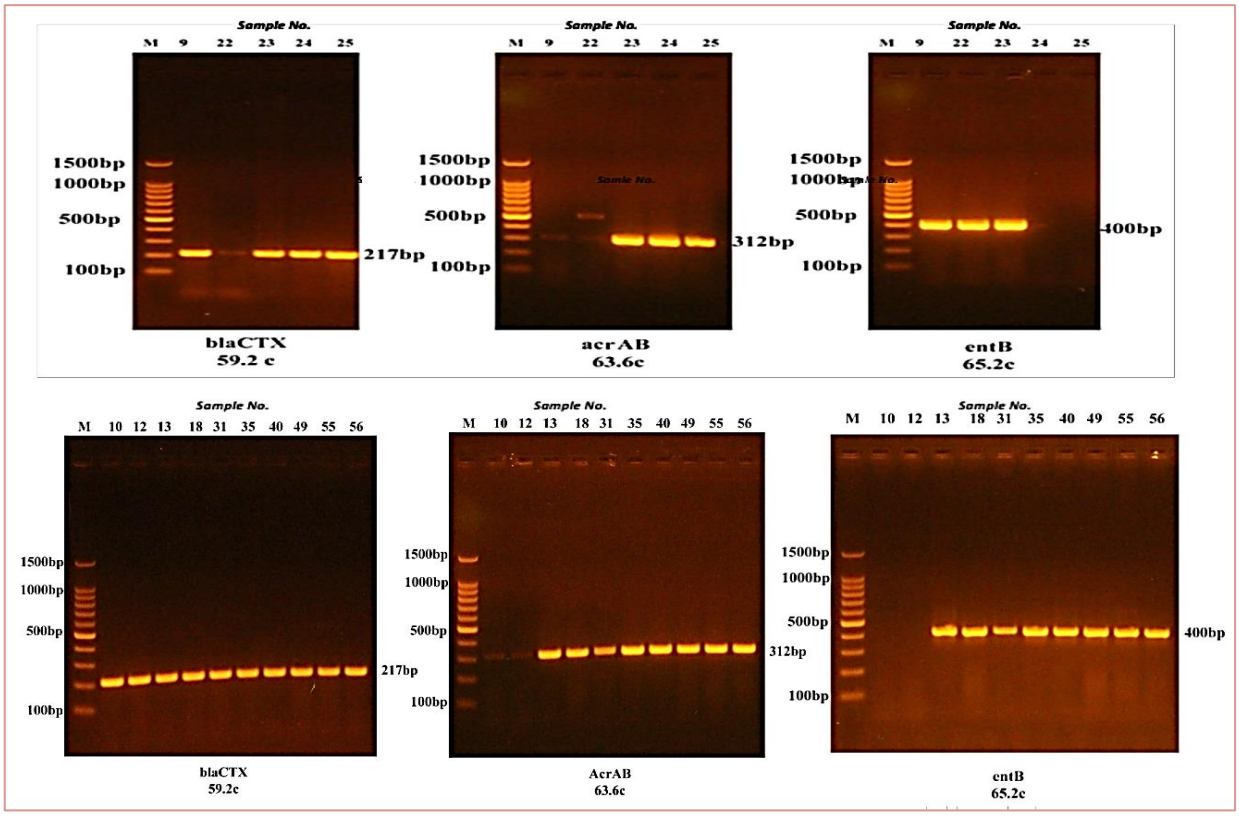

FIGURE 4: Uni-plex PCR amplification fragments for the detection of blaCTX gene (217bp), of acrAB gene (312bp) and of entB gene (400bp). M: (100-1500)bp DNA ladder. Amplicons were electrophoresed on agarose gel (1.5%) at 150 V/cm for 45 min, stained with Ethidium bromide, and visualized using a UV transilluminator documentation system

TABLE 6: The antibiotics that each of the K. pneumoniae isolates were sensitive to, the antimicrobial resistance phenotypes, and the amplified virulence genes for each isolate (+:present & -:absent).

Sample Specimens.	LEV 5	CIP 5	CN 10	SXT 1.25	AMC 30	IPM 10	CFM 5	FOX 30	ATM 30	FEP 30	AMP 10	CAZ 30	YA	HA	ST	magA	Aerobactin	mrkJ	mrkA	AcrAB	blaKPC	blaCTX	entB
9Bl	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	+	-	-	+	-	-	-	+	+
10Sp	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	+	-	+	+	-	-	-	+	-
12Ur	R.	R.	R.	R.	R.	I.	R.	R.	R.	R.	R.	R.	+	-	-	-	+	+	-	-	-	+	-
13Sp	I.	R.	R.	R.	R.	S.	R.	S.	R.	R.	R.	R.	+	-	+	+	-	+	-	+	-	+	+
18Ur	I.	R.	R.	R.	S.	R.	R.	R.	R.	R.	R.	R.	+	-	-	-	+	+	-	+	+	+	+

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22Sp	S.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	+	-	-	-	+	+	-	-	-	+
23Pe	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	+	-	-	+	-	+	-	+	+
24Bl	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	+	-	-		-	+	-	+	-
25Sp	R.	R.	R.	R.	R.	S.	R.	R.	R.	R.	R.	R.	+	+	-	-	+	+	+	+	-	+	-
31Pe	R.	R.	R.	R.	I.	R.	R.	S.	R.	R.	R.	R.	+	-	-	-	+		-	+	-	+	+
35Sp	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	+	+	-	-	+	+	+	+	+	+
40Ct	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	+	-	+	+	+	+	+	+	+
49Ur	R.	R.	R.	R.	R.	S.	R.	R.	R.	R.	R.	R.	+	-	-	-	-	+	+	+	-	+	+
55Wu	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	R.	+	-	-	-	-	+	-	+	-	+	+
56Ur	R.	R.	R.	R.	R.	I.	R.	R.	R.	R.	R.	R.	+	-	-	-	-	+	+	+	-	+	+
	MDR%															Frequency %						
	80.0	100	100	100	86.7	66.7	100	86.7	100	100	100	100	100	20.0	46.7	6.67	40.00	86.67	40.00	73.33	20.00	93.33	73.33

Abbreviation: YA: Yeast Agglutination, HA: Haem Agglutination, ST: String Test, CS: Clinical Specimens, MDR = multi-drug-resistant.
Amoxicillin-clavulanate (AMC), Ampicillin (AMP), Aztreonam (ATM), Cefepime (FEP), Cefixime (CFM), Cefoxitin (FOX), Ceftazidime (CAZ), Ciprofloxacin (CIP), Gentamicin (CN), Imipenem (IPM), Levofloxacin (LEV) and Trimethoprim-sulfamethoxazoile (SXT).
Sp: Sputum, Ct: Catheters swab, Es: Endotracheal tubes swab, Ws: Wound swab, Bl: Blood, Ur: Urine, Ps: Pus exudative.

Enterobactin (entB) and aerobactin (iucC), two iron uptake factors, have been characterized as virulence factors in Klebsiella pneumoniae strains.(54) Our analysis found that the majority of both hvKP and cKP isolates carried the entB gene, which is consistent with the bulk of earlier studies. (55),(54),(56),(57) Moreover, a connection was discovered between the aerobactin gene and the mucous regulating genes in K. pneumoniae. Given that both genes are present on the same plasmid, this relationship is not unexpectedtg.(58) Several previous studies have also reported entB as the most common siderophore detected in K. pneumoniae. (59),(60),(61) The prevalence of magA (mucoviscosity-associated gene A and specific to K1 capsule serotype) in our study was very low, which agreement with Leu et al. (16.7%) (62)

The efflux pumps may lessen the amount of antibiotics present inside of cells, which is crucial for bacterial survival. (63), (64) The AcrAB efflux pump was found in the majority of K. pneumoniae strains in our study. It had a strong correlation with the MDR phenotype.

Our results support the accounts of Mirzaie and Ranjbar, research showed that antibiotics, particularly fluoroquinolones like ciprofloxacin and beta-lactam (such as Ampicillin, Aztreonam, Cefepime and Ceftazidime antibiotics in MDR isolates), are metabolized by the multidrug efflux pump system (AcrAB) in K. pneumoniae strains (see table 6). (65)

In MDR strains, the genes linked to virulence were as well predominate. The most prevalent bacterium cells adhesive agents that allow K. pneumoniae to adhere to the epithelial and endothelial cells of the urinary tract and cause urinary tract infection are type I fimbriae and type III fimbrial adhesion (mrkA and mrkD). (66). Research have demonstrated that type III fimbrial attachment (major pilin subunit (mrkA)) is crucial for the development of biofilms in K. pneumoniae, however its precise mechanism is still unknown. (67).

Mirzaie and Ranjbar investigated the rate of biofilm development in K. pneumoniae strains, and their findings correlated with our study's finding that 40% of the strains were biofilm-

constituting. (65) The mrkA were found in all types of sputum and urine isolates in our investigation.

From the data presented, mrkJ represents a c-di-GMP-specific phosphodiesterase (PDE) that has pronounced negative effects on K. pneumoniae biofilm formation by c-di-GMP hydrolysis.(68)

The occurrence of co-carried blaKPC gene in a single bacterial strain in hospitals has raised concerns since it frequently results in an isolate that is particularly drug-resistant. (62)

Clinical diagnosis and therapy are significantly restricted by the hydrolysis of carbapenems and the majority of other β-lactam antibiotics by bacteria that produce carbapenemase. The majority of Carbapenemase-Resistant Enterobacteriaceae strains produce Klebsiella pneumoniae carbapenemase (KPC), which is a main category of carbapenemase (CRE). In recent times, the blaKPC gene has frequently been discovered in CRE strains. (69),(70),(62),(71)

Moreover, the results of the gene blaCTX-M that involved in ESBL resistance showed the prevalence of 93%, these results agreed with the reports of studies by Ahmadi et al., (72) in which the gene blaCTX-M frequency was 96%.

Similar findings were found in several papers, with blaCTX-M being the most common gene, followed by blaSHV and blaTEM(1),(73). Due to the widespread use of cephalosporins, especially Cefepime and Cefixime, there may be selection pressure contributing to the high incidence of blaCTX-M in K. pneumoniae.

In this investigation, 15 K. pneumonia strains were recovered, of which 3 were discovered to be blaKPC. All strains showed remarkable resistance to almost strong biofilm development, which is consistent with Liu et al, study (62). The significant biofilm production of pathogenic bacteria, which are frequently involved in hospital infections and always result in the failure of antibiotic therapies, makes this a risky state for antibiotic treatment.(70)

Study the effects of cinnamic acid and clove oil on gene expression of mrkA and mrkJ genes

To further confirm the inhibitory effects of the cinnamic acid and clove oil on the K. pneumoniae, which is strong biofilm-producing and their biofilm-associated genes (mrkA and mrkJ) were determined by quantitative RT- PCR. From the software of quantitative RT PCR, folding values of gene amplification were recorded. High folding values mean high gene expression, while low folding values show low gene expression. The calculation of gene expression fold change was done from the ΔΔCt value.).

This study showed that the down-regulation of type III fimbria (mrkA) biosynthesis gene and Phosphodiesterase (mrkJ) responsible for biofilm development after treatment with cinnamic acid and clove oil, except in case of mrkJ gene which treated by cinnamic acid that lead to up-regulation. As shown in table (7).

This chemical compound exerted its antibacterial effect by disrupting cellular metabolism and destroying cell membranes. Meanwhile, this compound could inhibit biofilm formation and decrease toxin secretion and infection risk (74).

TABLE 7: Gene expression of mrkJ and mrkA gene before and after treatment of K. pneumoniae with cinnamic acid and clove oil

Phytochemicals	mrkJ gene					mrkA gene				
	Δ Ct treated	**Δ Ct Control**	**ΔΔ Ct**	**Folding**	**Conclusion**	**Δ Ct treated**	**Δ Ct Control**	**ΔΔ Ct**	**Folding**	**Conclusion**
Cinnamic acid	-0.39	-0.11	-0.28	1.214	Up-R	-0.03	-0.11	0.08	0.946	Down-R
Clove oil	1.6	0.04	1.56	0.339	Down-R	2.02	0.04	1.98	0.253	Down-R
Control				1.000					1.000	
Abbreviation: Up-R: up-regulation, Down-R: down-regulation.										

CONCLUSIONS

The findings from this investigation showed that cinnamic acid and clove oil are powerful antibacterial agents against K. pneumonia's sessile and planktonic cells. Overall, clove oil & cinnamic acid was found to be more influential in preventing initial cell attachment contrasted to preformed biofilms. The metabolic activity of the biofilms is shown to be greatly reduced by the phytochemicals. The incorporation of clove oil and cinnamic acid in innovative disinfection and sanitizer formulations would be made possible by the identification of the bioactive components in these substances.

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