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Inhibitor Effect of *Cinnamomum cassia* Hexane Extract- Chitosan Nanoencapsulated on *fimA* and *mrkA* Biofilm Genes in *Klebsiella Pneumoniae* Isolated from Urinary Tract Infections

[1]Zeyad SH. Abbas, [2]Emad H. Jassim, [3]Hameed M. Jasim

[1]Ministry of Agriculture.
[2] Institute of Genetic Engineering and Biotechnology for post graduate studies, University of Baghdad, Baghdad, Iraq.
[3]College of Biotechnology, Al- Nahrain University.

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Abstract: Among the phytochemicals extracted from *Cinnamomum cassia* bark using hexane, which revealed the presence of terpenoids, alkaloids, flavonoids and phenols. Once 100 Klebsiella isolates from 170 UTI samples were morphologically cultured, only 60 *Klebsiella pneumoniae* isolates could be identified using conventional bacteriological and morphological methods. A highly pure RNA sample was collected. The three *K. pneumoniae* isolates were tested for *fimA* and *mrkA* gene mRNA expression compared with *rrsE* gene (housekeeping) using RT-qPCR. The RT-qPCR results show that when exposed to subMIC CHEx and CHCsNPs, respectively, the expression of the *fimA* gene significantly decreased ($p \geq 0.05$) as compared to the control group. This study utilizes quantitative RT-qPCR screening of the *mrkA* gene to uncover variations in gene expression among three distinct *K. pneumoniae* isolates. The *mrkA* gene Ct value ranging from 6.0 to 8, and did not differ statistically according to the analysis. *K. pneumoniae* bacteria secrete a many toxin, because the *mrkA* gene was not strongly expressed.

Keywords: Inhibitor, CHCsNPs, biofilm, *mrkA, fimA,* genes express, *K. pneumoniae*.

Corresponding author: (E-mail: zyad.shalal78@gmail.com).

Introduction

The antibacterial activity of plant extract/chitosan nanoparticles is attributed to the interactions between the nanoparticles and the bacterial cell wall or membrane, and these interactions involve the electrostatic attraction between the positively charged amino groups of glucosamine and the bacterial cell membranes (carrying a negative charge), this interaction leads to significant changes in the cell surface, a change in the ability of substances to pass through the cell membrane, an imbalance in the movement of water across the cell membrane, and the release of substances from within the cell, these effects ultimately result in the death of the cell. Additionally, this interaction has positive characteristics such as being readily available, compatible with living organisms, capable of breaking down naturally, not causing harm, and being accessible to modify chemically(1). The development of antimicrobial resistance in biofilm-forming isolates adds to the long-term survival of bacteria, potentially leading to persistent infections and therapeutic

challenges (2,3). *K.pneumoniae* typically exhibits a smooth lipopolysaccharide (LPS) with an O antigen and a capsule polysaccharide (K antigen) on its surface. Both of these components contribute to the development of diseases caused by this species of bacteria (4). *K. pneumoniae* strains possess various virulence factors that enable them to invade the host. These factors include capsular polysaccharides, lipopolysaccharide, serum resistance, urea, enterotoxin production, type 1 and type 3 adhesions factors involved in aggregative adhesions, and siderophores (5). The bacteria is present in the commensal gut flora and is also frequently found as an opportunistic pathogen in intestine, urinary tract, and bloodstream infections. They often possess ESBL-encoding genes. *K. pneumoniae* strains that possess the ability to manufacture extended- spectrum β- lactamases (ESBLs) exhibit significant activity against a wide range of β-lactam drugs. Furthermore, these highly infectious strains can develop resistance against several types of antibiotics other than β-lactams, which pose a challenge in treating infections; these strains are commonly known as multidrug-resistant (MDR) strains (6). Cassia, or Chinese cinnamon, is an aromatic, medium-sized, evergreen tree native to southern China's lowlands and planted across Southeast Asia. Due to its scent, cinnamon is utilized in aroma and essence industries to add to meals, perfumes, and medicines (7). *Cinnamomum cassia* has yielded around 160 chemicals. Cinnamaldehyde and trans-cinnamaldehyde (Cin) are the main ingredients of cinnamon essential oil, contributing to its smell and biological activities (8). Aim the study to evaluate the activity of *C. cassia* hexane extract/ chitosan nano-encapsulation against *K. pneumonia mrkA, fimA* genes expression reposing on Adherences and biofilm- producing activity.

Material and methods

Preparation of cinnamon extracts

The Department of Biology/ College of Science, University of Baghdad has discovered the *Cinnamomum cassia* bark that was obtained from local market places in Baghdad. The hexane solvent was used to hexane extract cinnamon bark, following the procedure of (9) with certain modifications. The bark was extracted by employing distilled n-hexane in a Soxhlet device for 8 hours. Following filtration, the extract was subjected to evaporation under decreased pressure at a temperature of 40 °C using a rotary-type evaporator. This process resulted in the production of a concentrated cinnamon extract in hexane, which was then kept at a temperature of -20 °C until it was required.

Evaluation of phytochemicals groups in cinnamon oil extract

The presence of bioactive groups (such as tannins, resins, Coumarins, saponins, alkaloids, phenols, terpenoids, flavonoids, glycosides, and steroids) was evaluated through the screening of cinnamon extract's preliminary qualitative phytochemicals according to the procedures outlined in (10).

Biosynthesis of cinnamon hexane extract –chitosan nanoparticles by sol gel method

For 60 minutes at 50 °C in filtrated water, ten milliliters of a solution containing 1% organic chitosan (Sigma Chemicals) dissolved in acetic acid was combined with ten milliliters of cinnamon hexane extract (CHE), the freeze-drying process for cinnamon

hexane extract/ chitosan nanoparticles (CHCNPs) following centrifugation. The CHCNPs were subsequently characterized using a variety of techniques, including a UV- VIS spectrophotometer, Fourier Transform Infrared Spectroscopy (FTIR), scanning electron microscopy, transmission electron microscopy, energy destruction X-ray (EDX), and atomic force microscopy (AFM). These methods were also integrated with the transmission operation mode of the(11).

Klebsiella isolates preparation

There were 60 isolates found to be infected with *K. pneumoniae* bacteria. We obtained (Urinary Tract Infections) patient isolates from various Baghdad hospitals, including Al-Karama and Yarmouk teaching hospitals, from Jun to October/ 2023. The isolates cultured on blood agar MacConkey and Eosin methyl blue agar and incubated at 37°C for the entire night. The initial identification of the colony was based on its phenotypic features, which included size, colour, shape, and mucus after a pure culture of isolated bacteria was obtained. After staining with gram stain, it was determined by microscopic inspection(12). To document the results, gram-negative bacteria were isolated, and their expanding colonies were observes.

Biochemical tests

A several of biochemical tests was performed on the isolates to confirm their authenticity. We conducted all test adhering to the guidelines outlined in 1996(13). In order to verify the isolates' identification, a battery of biochemical tests was administered to them (Test for Indole). The tubes were filled with newly formed colonies and left to incubate at 37 °C for one day, two drops of Kovac's reagent were added to each tube. Catalase Assay: A drop of 3% hydrogen peroxide was added after the colony was placed on a microscope slide using a wooden stick. Examination of Oxidase Activity: Using a sterile wooden brush, one colony of each isolate was distributed on the filter paper. Test for Motility: After inoculating cultures with a straight wire into the tube's center and incubating them at 37ºC for 24 hours, a motile organism was detected when they moved away from the stabbing line or when cloudiness formed around the stabbing area. Evaluation of the Urine: Following a 24-hour incubation period at 37°C, the sterile loop was used to streak the previously prepared urea agar with an infection. Evaluation of Lactose Fermentation: We cultured the studied bacterium for 24 hours at 37°C after infecting McConkey with it using a sterile loop on a medium.

Determination of the inhibition activity of CHEx and CHCsNPs against *K. pneumonia* biofilm gene expression

Using the TransGen, biotech. ER501-01 TransZol Up Plus RNA Kit's components (Trans Gen biotech. China). Total RNA was extracted from all samples using the TransZol Up Plus RNA Kit Reagent according to the manufacturer's instructions. The concentration and purity of the extracted RNA were evaluated using a Thermo Fisher Scientific, USA, 2000c Nanodrop spectrophotometer to evaluate the quality of the samples for RT-qPCR analysis. The amount of RNA for the samples ranged between 82 and 164ng/ µl, and their absorbance was measured at two different wavelengths (260 and 280 nm) to confirm the purity of RNA. If the A260/A280 ratio was around 2.0, it meant the RNA sample was pure. The cDNA synthesis reaction component,

Easy Script® One-Step gDNA Removal and cDNA Synthesis Super Mix (Trans Gen, Ref. AE311-02), is used to reverse-transcribe to complementary DNA. The reaction must be conducted in a volume of 20 µl according to the instructions provided by the manufacturer. It was necessary to reverse transcribe 4 microliters of total RNA. A 15-minute incubation period at 42°C was used for qPCR with anchored oligo (dT) 18 primer and GSP. Ten minutes at 25°C were used incubate a randomly selected primer. Enzymes are rendered inactive by incubating them for five seconds at 85ºC.

The expression levels of *fimA, mrkA, rrsE* genes

We measured the expression levels of the *fimA* and *mrkA* genes using the sensitive, quantitative polymerase chain reaction (RT-qPCR) method for assessing steady-state mRNA levels. An experiment using quantitative real-time RT-qPCR SYBR Green was conducted to confirm the expression of the target gene. Alpha DNA Ltd. (Canada) built the primer sequences for the *fimA* and *mrkA* genes, which were then lyophilized and stored at -20°C. By increasing and utilizing the endogenous regulatory gene *rrsE*, the mRNA levels of the *fimA* and *mrkA* genes are normalized. Primers for the *rrsE* gene, *fimA* gene, and *mrkA* gene respectively are as follows: F-TTGACGTTACCCG CAGAAGAA, R-TCTACAAGACTCT AGCCTGCCA (187 bp), F- CATCCG CGTTCGCTATACCA, R-TTCTGGCC CTGCAAAACTCT (210 bp), F- ACGT CTCTAACTGCCAGGC, R-TAGCCCT GTTGTTTGCTGGT (115 bp). Quick real- time polymerase chain reaction, or RT- qPCR, also known as qPCR, the QIAGEN Rotor gene is utilized in Germany in real time PCR. The *rrsE*, *fimA*, and *mrkA* genes' expression levels were assessed using the TransStart® Top Green qPCR Super Mix kit. The fold changes were determined by measuring the threshold cycle (Ct). We repeated each response twice. The initial approach for calculating fold variations of the quantitative expression of mature RNAs, which was published by (14), is the relative cycle threshold (Ct) ($2^{-\Delta Ct}$). The relative gene expression ratio compares the two groups (control and test) and finds the mean. When the value is larger than 1, gene expression is up-regulated; when it is between 0 and 1, gene expression is down-regulated, and when it is equal to 1, there is no change. In order to generate reliable Ct values from the RT-qPCR equipment, the expressions of the target genes were normalized by establishing suitable thresholds.

The term refers to the relative gene expression ratio in the group compared to that in the control group. The *rrsE* gene was used as the housekeeping reference gene, whereas the *fimA* and *mrkA* genes were assessed using the double delta Ct technique. The calculations. The threshold cycle (CT) was determined for each sample using the real-time cycler programme. The average values of each sample were found by performing repeated runs for each one. The Ct values for the target genes (being assessed in patients and controls) and the housekeeping genes *rrsE*, *fimA*, and *mrkA* were recorded. The ΔCt, which represents the difference between the Ct values of the target gene and the housekeeping gene, was calculated by subtracting the selected normalization factor from the Ct value of each gene of interest. This ΔCt is also referred to as the "normalized raw data."

ΔCt = CT of target gene – CT of house-keeping …….. (1)

ΔΔCt = ΔCt (treated)- ΔCt (Control) …….. (2)

Finally, the expression ratio was calculated using the following formula:

$2^{-\Delta CT}$= Normalized expression ratio……. (3)

Results and discussion

Extraction yield of *Cinnamomum cassia*

The hexane extraction of phytochemicals from *C. cassia* bark resulted in a satisfactory yield of 1.7% cinnamon bark powder. The n-hexane extract of *C. cassia* bark was subjected to phytochemical screening, which detected the presence of phenols, flavonoids, alkaloids, and terpenoids. Flavonoids were present in moderate amounts, while tiny proportions of phenols, alkaloids, and terpenoids were also detected, as indicated in (Table1). Secondary metabolites, known as active groups witch play a vital role in plants' defense mechanisms against insects, bacteria, and other creatures. Active organizations have also provided significant advantages to humanity in several domains, including the realms of food and medicine. The investigation confirmed the presence of all active components in the oily extract of *C. cassia* extracts, specifically cinnamon bark, albeit in varying quantities.

Table (1) : Phytochemical screening of *n*-hexane extract of *C. cassia* bark.

Active Component	n-Hexane extract of bark
Tannins	+
Resins	+
Coumarins	+
Saponins	+++
Alkaloids	+
Phenols	+
Terpenoids	+++

(+++) high amount after added of reagent immediately (++) ; moderate amount after 5 min of reagent added; (+) low amount after 10 min of reagent added.

Identification of active compounds in the hexane extract of *C. cassia* showed that most of the biological activity of aromatic and medicinal plants comes from the active compounds that make up those plants. The function of these compounds indicates that they possess potent medicinal properties. There are many types of secondary metabolites. The presence of these metabolites is one possible explanation for the antimicrobial and antibacterial properties of the plant extracts. Many substances actively combat harmful microorganisms (15).

Preparation and characterization of *K. pneumoniae* isolates

All of the urine samples included bacterial growths that had been cultured in enrichment media for the purpose of identifying *K. pneumoniae* isolates using conventional morphological and bacteriological culture techniques. After incubating for 24 hours at 37°C, the colonies of K. pneumoniae on MacConkey agar took on the characteristics of a big, mucoid, convex, circular organism and a pinkish hue as a

result of the fermentation of lactose sugar. On blood agar, they show up as mucoid colonies that aren't hemolytic and grey-white. The findings corroborated those of(16).

Culturing and Biochemical Examination

After morphologically cultivating 100 Klebsiella isolates from 170 UTI samples on blood agar, MacConkey agar, and selective media, the colonies were incubated at 37 °C for 24 hours as part of the culturing evaluation. based on morphological and cultural characteristics 60 *K. pneumoniae* isolates were lactose-fermented and pink; at the same time the (17, 18, and 19) identified 30.4% and 27.28% respectively. The distinctive colour of the bacterial colonies was caused by the degradation of synthetic substrates in the medium, which the bacteria broke down using enzymes they produced during metabolism. The colonies that were recovered the night after inoculation may be detected by colour(19).

As illustrated in (Table 2) a battery of biochemical assays was initially performed to detect *K. pneumonia*. Colonies treated with hydrogen peroxide reagent showed gas bubble formation in the majority of the suspect isolates, suggesting a positive catalase test. But the oxidase test came out negative. Despite a positive urease test, the isolates failed to produce indole or exhibit motility, according to the biochemical testing, together with the beneficial fermentation of lactose. In this study, we compared the conventional biochemical findings to the gold standard published by (12). To begin, as shown in (Table 2), a battery of biochemical tests was run to detect the existence of *K. pneumonia*. The current investigation contrasted the conventional biochemical findings with the gold standard results given by (20,21).

Table (2): Morphological and Biochemical Identification Results of *K. pneumonia* isolates.

No	Biochemical Test	Result
1	Gram stain	-
2	Catalase production	+
3	Oxidase test	-
4	Indole production	-
5	Urease production	+
6	Lactose fermentation	+
7	Motility test	-
8	Microscopic shape	Road shape

Determination of the inhibitor activity of CHEx and CHCsNPs against *Klebsiella pneumoniae* biofilm gene expression

Escherichia coli and *Klebsiella pneumoniae* are both implicated in opportunistic infections and pathogenic processes in both veterinary and human medicine. This includes their ability to produce biofilms, as discussed by (21). The process of microbial adherence to surfaces and subsequent biofilm formation is widely recognized as a significant issue due to its considerable economic and public health implications across various human sectors. Pathogenic bacteria found in

healthcare facilities pose a significant health danger to customers (22,23).

Gene expression analysis by using quantitative reverse transcriptase real- time RT-qPCR technique

RNA concentration and purity assessment

The RNA concentration varied between 82-164 ng/ µl, indicating a range of RNA amounts present. The purity of the RNA sample was approximately 2.0, suggesting a high level of purity.

The activity of CHEx and CHCsNPs against *fimA* gene expression in *K. pneumoniae* isolates

The objective of this phase is to quantify the expression of *fimA* and *mrkA* genes and compare the gene expression in the presence of CHEx, CHCsNPs, or no therapy. RT-qPCR stands out from other gene expression assays due to its precision, sensitivity, and rapidity in producing data. In a relative quantification study, the focus is typically on comparing the expression level of a specific gene across various samples (24). It is crucial to understand this. The current work utilized an RT-qPCR method to assess the mRNA expression of *fimA* and *mrkA* genes. Treatments were selected based on submitting levels for comparison. The amplification was quantified using the Ct value, with the housekeeping gene *rrsE* being employed. This gene is commonly used in molecular research due to its consistent expression across multiple settings, as seen in (Figure1).

The amplification was measured and recorded as the Ct value, which represents the level of gene expression. A high Ct value implies low gene expression, whereas a low Ct value suggests strong gene expression. Calibrator genes, which are sometimes referred to as housekeeping genes, are used in molecular research under the assumption that their expression remains constant in the cells or tissue being investigated (25). The precision of RT-qPCR findings heavily relies on the choice of reference genes (26). The requirement for the accurate application of RT-qPCR to analyze changes in target gene expression is the validation of its validity. Previous research has utilized the *rrsE* gene as a housekeeping gene (27). No discrepancies were seen in the gene amplification pattern between the isolates prior to and following treatment with CHCsNPs. The melting temperature of (Figure 1) ranged from 76°C to 78°C. The *rrsE* gene, which serves as a genetic marker for bacterial species identification, is found in all members of this domain (28). In this study, three *K. pneumonia* isolates were analyzed using quantitative RT-PCR to determine the mRNA expression of the *fimA* gene. The isolates ranged in Ct values from 16.7 to 17.61, 17.02 to 17.36 and 17.54 to 17.81. These findings corroborate those of earlier research (29, 30). found that the *fimA* gene, which encodes type1 fimbriae, can aid in bacterial adhesion and biofilm formation. The results showed that every single *K. pneumoniae* isolates tested positive for the presence of the *fimA* gene. (31) also found the presence of the *fimA* gene in 100% of samples, so our results are in agreement with his. Having an increased CT value in CHCsNPs produced by biosynthesis is indicative of low expression of the *fimA* gene (fold), which is responsible for the secretion of toxins of *K. pneumoniae* isolates.

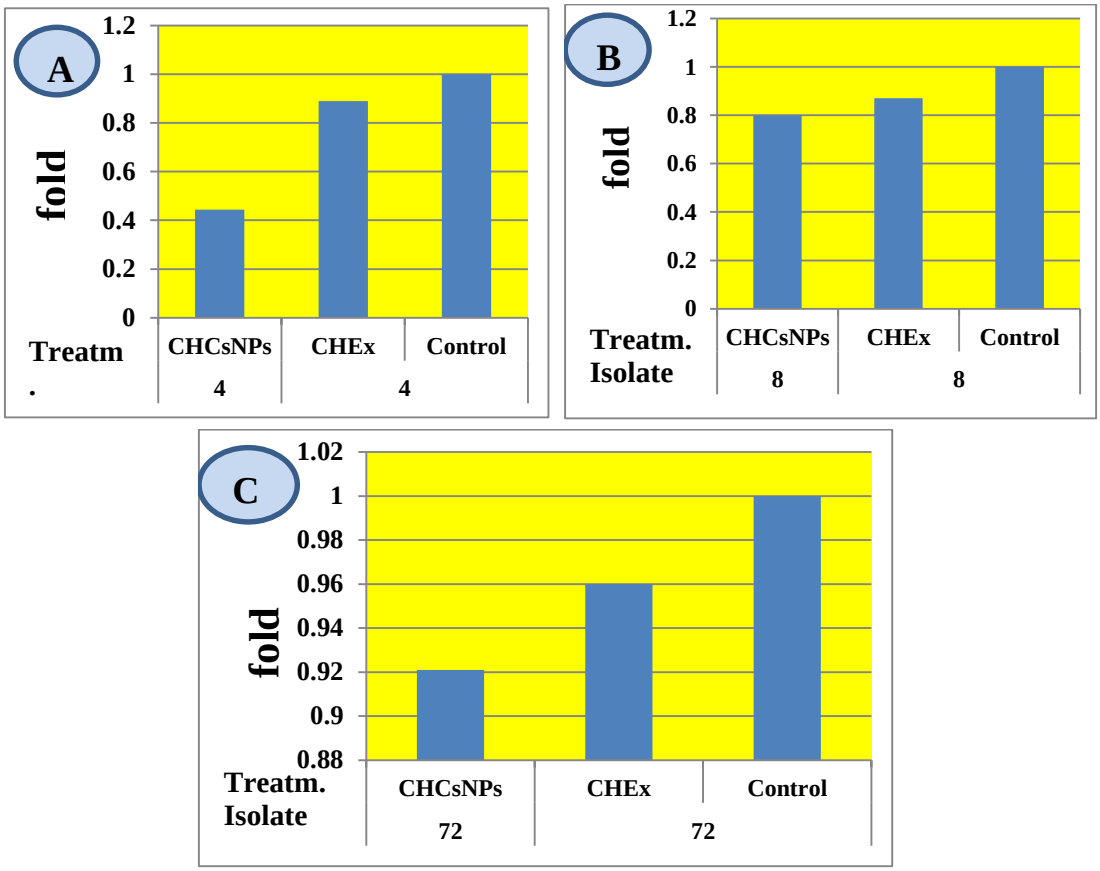

Figure (1): Fold of *fimA* gene expression in 3ed *K. pneumonia* isolates A (isolate 4), B (isolate 8), and C (isolate72) were treated with CHCsNPs and CHEx Depending on the $2^{-\Delta Ct}$ Method.

By comparing three *K. pneumoniae* isolates to the control group, the RT-PCR results reveal a substantial decrease ($p \leq 0.05$) in the expression of the *fimA* gene when exposed to subMIC CHEx and CHCsNPs, respectively. No significant alterations were seen with other isolates' *fimA* genes.

The activity of CHEx and CHCsNPs against *mrkA* gene expression in *K. pneumonia* isolates.

This study reveals differences in gene expression across three different *K. pneumoniae* isolates using a quantitative RT-PCR assay for the *mrkA* gene. For all isolates except 4, 8, and 72, there were statistically significant variations ($P \leq 0.05$) in the CT value among treatments. The *mrkA* gene's Ct values ranged from 5.950 to 12.860 for isolate 8 and from 6.170 to 16.330 for isolate 72 (Figure2). Several factors contributed to this, such as the sources of the isolates, the places of research, the hygiene restrictions, and the methods used for the assay (32). However, statistical analysis revealed that there were no significant differences ($p \geq 0.05$) between the treatments for isolate 5, which had a range of Ct values of the *mrkA* gene from 6.0 to 8.7. showed that the *mrkA* gene was not highly expressed, which meant that the *k. pneumoniae* bacteria were not secreting as many toxins (33) and found similar outcomes in their research.

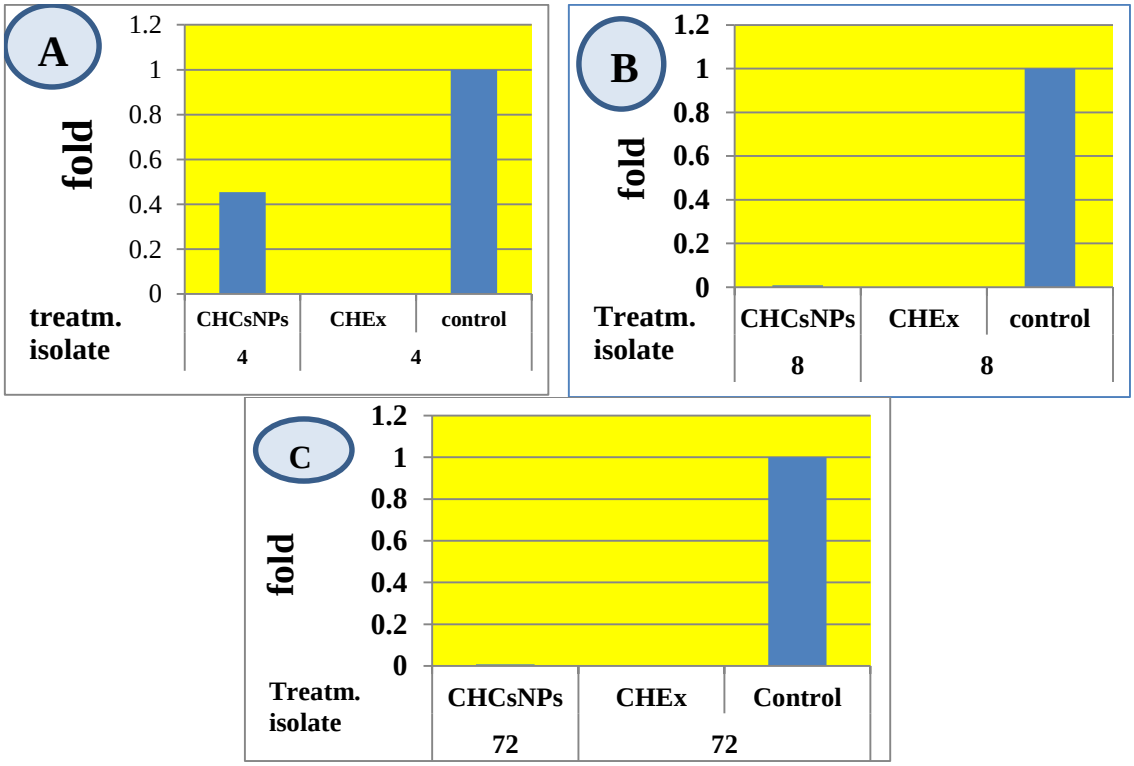

Figure (2): Fold of *mrkA* gene expression in 3ed *K. pneumonia* isolates A (isolate 4), B (isolate 8), and C (isolate 72) were treated with CHCsNPs and CHEx Depending on the $2^{-\Delta Ct}$ Method.

In order to determine how phytochemicals and organic acids, both in extract and nanoparticle form, affect the ability of *K. pneumonia* to invade pig cells, our research demonstrates that cinnamaldehyde and carvacrol limit *K. pneumoniae* penetration into epithelial cells in a way that is distinct from bacterial or cell toxicity, and that this impact is amplified when specific organic acids are present. Kuźmińska-Bajor *et al.* (34) discovered that neither the test substances nor any other factors found *in vivo* interacted with the flora. Cinnamaldehyde and carvacrol may interact with these acids, although there is limited information available on this. Cinnamaldehyde and cinnamic acid are two examples of phytocompounds that react poorly with acids; carvacrol and cinnamic or propionic acid are two more examples. The damaged cells experience more inflammation when there is an increase in the release of fimbrial proteins(35). Type 1 fimbriae synthesis is upregulated in response to bacterial stress, according to (36). However, this upregulation does not lead to enhanced invasion but rather to a decrease in the bacteria's capacity to infiltrate host cells. The cinnamon hexane extract-chitosan nanoparticles, which contain biologically active compounds like cinnamic acid, eugenol, and cinnamic aldehyde, are responsible for the decrease in *fimA* and *mrkA* gene expression. The CHEx demonstrated antibacterial activity against *K. pneumoniae*, and the electrostatic force between the chitosan and bacterial cell wall promoted a closer interaction with charged molecules, allowing CHCsNPs to penetrate the bacterial cell wall. The bacterial cell death cascade begins with

this interaction, which causes surface modifications to the cell and changes the permeability of the cell membrane. This causes an osmotic imbalance and the efflux of intracellular substances, which in turn bind to DNA and disrupt DNA replication. Previous studies by (29, 36) corroborate these findings. while the (37) results showed that strong biofilm production isolates had the highest gene expression, in contrast the isolates that showed moderate and weak biofilm production recorded the lowest gene expression.

Conclusion

The results demonstrate that at concentrations insufficient to influence bacterial growth, viability, motility, or fimbria production, cinnamaldehyde, carvacrol, cinnamic, and propionic acids reduce *k. pneumonia's* capacity to invade UTI cells. These trace amounts alter gene expression in UTI cells and, in rare instances, produce type 1 fimbriae abnormalities. Many chemicals have demonstrated antimicrobial action, and there has been growing interest in using organic acids and essential oils as new antimicrobial agents. But we need to determine if these chemicals might increase inflammation and if they can also stress out the bacteria's host. One example is cinnamaldehyde, which causes UTI cells to become inflamed and stressed. In the absence of propionic acid and subsequent exposure to *K. pneumonia*, carvacrol failed to cause cellular inflammation.

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