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Food Additive Mediated Biosynthesis of AgNPs with Antimicrobial Activity Against Hypermucoviscous Enterotoxigenic Foodborne *Klebsiella pneumoniae*

Marwa H. AlKhafaji[1*], Raghdan H. Mohsin[2]& Ayoob M. Alshaikh Faqri[3]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
[2]Department of Animal Production, College of Agriculture, University of Basrah, Iraq
[3]Middle technical University, Balad Technical Institute, Baghdad, Iraq

***Corresponding author email**: **M.H.A.**: marwa.alkhafaji@sc.uobaghdad.edu.iq ; **R.H.M.**: raghdan.mohsin@uobasrah.edu.iq; **A.M.A.**: ayoob-murtadha@mtu.edu.iq

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Abstract: This study was aimed to adopt an ecofriendly method to synthesize nanoparticles with an effective antibacterial and anti-biofilm activity. A food origin hypermucoviscous Klebsiella pneumoniae was isolated from food samples and identified using biochemical tests and the Vitek system. A string test was depended on to identify hypermucoviscous isolates. Enterotoxicity of foodborne hypermucoviscous K. pneumoniae isolates was detected phenotypically using suckling mouse bioassay. Biofilm forming ability was tested for all the isolated bacteria using the Microtiter plate method and Congo Red Agar. A natural food additive *Syzygium aromaticum* (clove) aqueous extract was used for the biosynthesis of silver nanoparticles in optimized conditions. The biosynthesized clove-silver nanoparticles (Clove-AgNps) were characterized by several techniques, and their antimicrobial and antibiofilm activity was determined. The results of this study revealed that the isolation of 28 *K. pneumoniae* isolates were isolated from 200 food samples. String test results showed that 16/28 (57.14%) *K. pneumoniae* isolates were hypermucoviscous. Eleven of these isolates (68.75%) were enterotoxigenic. Using clove aqueous extract as a biological agent was successful in the biosynthesis of AgNPs with an average diameter of 14.12 nm as measured by AFM. The optimum biosynthesis conditions were: 1mM of AgNO3 concentration, pH 7, at 37 °C, and 24 hours. The minimum inhibitory concentration of the clove-AgNPs was detected as 62.5 µg.mL^{-1}. Sub-inhibitory concentration of 31.25 µg.mL^{-1} of Clove-AgNPs resulted in: 91% decrease in the formed biofilm. It can be concluded that using *Syzygium aromaticum* is an effective ecofriendly method for AgNPs biosynthesis with excellent antibacterial and anti-biofilm activity against enterotoxigenic hypermucoviscous *K. pneumoniae*.

Keywords: Antibacterial Agents, Biofilm, Clove, Food Poisoning.

Introduction

Uncontrolled and dual use of antibiotics has led to the emergence of multidrug-resistant (MDR) bacteria, which pose a significant hazard to the global population due to their infectious character (Hamida *et al*., 2020). *Klebsiella pneumoniae* is Gram-negative

bacteria that belong to the Enterobacteriaceae. It is capable of causing a variety of infectious diseases as urinary tract infections, bacteremia, and pneumonia, especially in immunocompromized patients (Lee *et al*., 2017; Wang *et al*., 2020). There are two pathotypes of *K. pneumoniae*, classical and hypervirulent (hvKp). A typical subtype of *K. pneumoniae* strains is the most prevalent one. hvKp was first described in Taiwan in 1986 (Choby *et al*., 2020); hvKp is characterized by the development of hypermucoid colonies on agar plates. String test was created and is employed as a phenotypic test for hypermucoviscosity (Wang *et al*., 2020). Early studies investigating hvKp for antibiotic resistance discovered very low frequencies of resistance, with less than 5% of hvKp bacteremia isolates generating extended-spectrum-β-lactamase (ESBL) (Li *et al*., 2014, Zhang *et al*., 2016). Such antibiotic-resistant isolates could spread and cause invasive illness in healthy people, which could have a significant impact. *Klebsiella* spp. was previously thought to be a normal element of the intestinal flora, and even when isolated from stool from people with diarrhea; these germs were not regarded as a causative agent of gastrointestinal infections. However, the first evidence that *K. pneumoniae* isolated from individuals with tropical sprue had the potential to secrete enterotoxin occurred in 1975 (Chang *et al*., 2021). Further research revealed that this enterotoxin can drive water and electrolyte secretion while also inducing structural alterations in the intestinal mucosa in a variety of species. Furthermore, heat-labile and heat-stable toxins were discovered in 1976 (Kienesberger *et al*., 2022).

Biofilm formation is one of the life forms of *K. pneumoniae*, these organized bulks of bacteria can survive in hostile environmental conditions (Guerra *et al*., 2022). Since it is difficult to treat infections caused by biofilm-forming bacteria, it is, therefore, necessary at this time to search for new biofilm inhibitors. There has been a serious need for the exploration of alternatives that could be used to treat multidrug-resistant microbial infections apart from antibiotics. Nanotechnology has energetically sophisticated to play a vital role in the novel research field with potential implications in biomedical technology (Ramos *et al*., 2017). The biosynthesis of nanoparticles has attracted a significant attention as it is less complicated and more sustainable green method of synthesis. Theses biosynthesized nanoparticles have better controllable sizes and shapes and elicits stronger antimicrobial activity, stronger that those created by conventional physical and chemical processes (Zhao *et al*., 2022). It is demonstrated that the size, shape, composition, crystallinity, and structure of silver nanoparticles (AgNPs) affect their properties. Due to their exceptional electro-catalytic activity, AgNPs are frequently used in a variety of biomedical and bio-electrochemical applications (Kulshrestha *et al*., 2016). Therefore, this research was designed for the synthesis of food additive-based AgNPs and to evaluate it as antibacterial and antibiofilm against enterotoxigenic foodborne hypermucoviscous isolates of *Klebsiella pneumoniae.*

Methods

Collection of food Samples

This study included a total of 200 food samples. The food samples were collected arbitrarily from local markets in Baghdad city (ALzubaidi & Alkhafaji, 2023). These foods included: 50 red meat, 50 chicken meat, 50 cheese, and 50 vegetable samples.

Bacterial isolation and identification

One gram of each sample of food was suspended in 9 mL distilled water (DW), and left for 20 min. at room temperature, then 1mL from each diluted sample was placed in the centre of the sterile Petri dish. The Petri dish holding the inoculum was then filled with cooled molten MacConkey agar, which was thoroughly mixed. The agar plate was inverted and incubated once it had solidified for 24 hours at 37°C. Then the grown colonies were further investigated to confirm identification. To identify the isolated bacteria both microscopic and macroscopic characteristics on selective and differential culture media in addition to the Vitek-system were depended to identify the isolated bacteria (Mahon *et al*., 2014).

Detection of hypermucoviscosity

A string test was used to identify hypermucoviscous isolates of *K. pneumoniae* (hkp). In this test a bacteriology inoculation loop was used to 'stretch' a colony away from the agar plate on which it was grown at 37°C overnight. The string test-positive was identified when a mucoid string > 5 mm was formed between the loop and the colony (Li *et al*., 2014).

Suckling mouse bioassay for detection of enterotoxin

The assay is designated for the detection of heat-stable enterotoxins. Firstly, crude enterotoxin was extracted by cultivating the strains in 20 mL of Tryptic soy broth (TSB) with 0.6% yeast extract then followed by incubation in a shaker incubator at 37°C with agitation at 120 rpm for 24 hours. Following that, the cultures were centrifuged at 10000 rpm for 30 min. at 4°C in sterile centrifuge tubes. The supernatant was then filtered through sterile 0.45 µm membrane filters, yielding a cell-free filtrate for use in the enterotoxin bioassay (Trower *et al*., 2000). Using a needle, 0.1 mL of culture supernatants were injected into the milk-filled stomachs of 1-3 day-old newborn mice weighing between 1-1.2 g. The quantity of fluid collected in the intestine was evaluated after three hours by calculating the intestinal weight to total body weight ratio. A ratio of 0.08 or more indicates the existence of heat-stable enterotoxin (Jay *et al*., 2005).

Detection of biofilm production

Biofilm formation by foodborne isolates of enterotoxigenic hkp was tested using two assays:

Congo red agar

Congo red agar (Freeman *et al*., 1989) was used to test the bacterial ability to produce a silme layer. This medium is composed of: brain heart infusion broth (37 $g.L^{-1}$), sucrose (50 $g.L^{-1}$), agar-agar (15 $g.L^{-1}$) and congo red dye (0.8 $g.L^{-1}$). All the constituents (except the dye) were mixed, heated and autoclaved, then cooled to 55 to 60°C. After that the congo red solution was added. A sterile Petri plate was filled with the prepared medium. Plates were inoculated with the test bacteria, and incubated aerobically at 37°C for 24 hours. A positive result was indicated by black colonies with a dry crystalline consistency.

Microtiter plate method

Microtiter plate assay (Coffey & Anderson, 2014) was used to detect the ability of hkp isolates for biofilm formation. Crystal violet (0.1%) w/v was used to stain the adherent bacteria developed on the polystyrene wells. Uninoculated TSB was added to the wells that were considred as negative control to determine the background optical density (OD). Micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm was used

to obtain OD of the stained adherent biofilm. The experiment was performed in triplicate and repeated three times and the average of OD values was calculated for the negative controls and all the tested strains. Cutt-off OD c value was estimated and the bacterial isolates were classified as in table (1):

Table (1): The biofilm formation equation (Hassan *et al*., 2011).

O. D. at 630 nm	Biofilm
OD ≤ OD c	non- biofilm producer
OD c <OD ≤ 2*OD c	weak biofilm-forming
OD c <OD ≤ 4*OD c	moderate biofilm producer
2*OD c <OD ≤ 4*OD c	strong biofilm-forming
4*OD c <OD	very strong biofilm-forming

* OD c were negative control wells

Biosynthesis of AgNPs using clove aqueous extract

Clove aqueous extract Preparation

Cold water extraction protocol (Singh *et al*., 2010) was modified in this study to achieve the aqueous extract of *Syzgium aromaticum* (clove). Clove extract was used as a reducing and capping agent for the biosynthesis of AgNPs. Clove aqueous extract was prepared by dipping 50 g of clove in a liter of deionized distilled water (DDW) for 48 hours. A filtration step was followed to remove the solid part.

Preparation of stock solution of silver nitrate **($AgNO_3$)**

A stock solution of $AgNO_3$ was prepared by dissolving 1.0 g of $AgNO_3$ in 100 mL of DDW and thus 10^{-3}M was ready to use in the reaction of biosynthesis.

Biosynthesis of clove-silver Nanoparticles (Clove-AgNPs)

The reaction mixture was 25 mL of Clove aqueous extract with 50 mL of 1Mm $AgNO_3$. Then it was incubated at 37°C in a rotary shaker at 120 rpm for 24 hours (green synthesis) (Divya *et al*., 2019). Usually, if the colour changed from light yellow to brown it indicates the formation of silver nanoparticles but in this experiment; the clove aqueous extract has a light brown colour. So the formation of dark brown colour indicated Clove-AgNPs formation. The synthesized clove-AgNPs were separated utilizing centrifugation at 10000 rpm for 15 min. The obtained pellet was left to dry in open air and then stored at $^{-}$4°C. The produced silver nanoparticles were purified by centrifugation at 10,000 rpm for 30 min (Gao *et al*., 2017). The pelleted AgNPs were centrifuged and washed three times with sterile DDW. The obtained nanoparticles were pure and quite stable then dried at 40°C (Fig. 1).

In this work, several parameters, that may have an impact on the quality of the nanoparticles were optimized. Including: pH, temperature, and $AgNO_3$ concentrations

$AgNO_3$ Optimization

Different $AgNO_3$ (1, 2 and 3 mM) concentrations were added to the clove exract and incubated for 24 hours. clove extract without $AgNO_3$ was kept as a control. Then the formed silver nanoparticles were characterized.

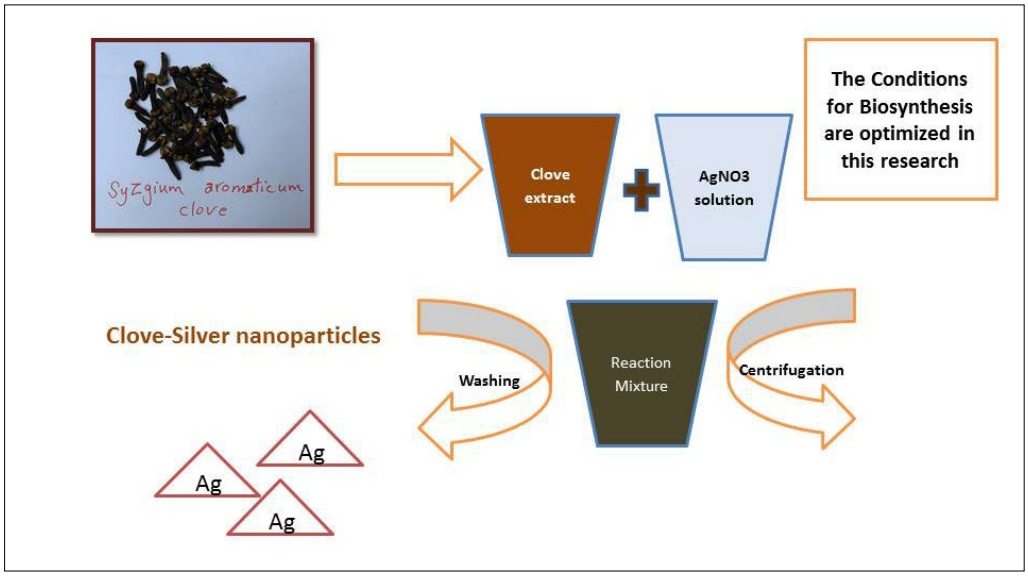

Fig. (1): Schematic representation for the Clove-AgNPs biosynthesis in this study

Optimization of parameters for the biosynthesis of Clove-AgNPs.

Temperature optimization

Different temperatures (4, 37 and 80°C) were selected. $AgNO_3$ was added to the clove exract and incubated for 24 hours at the selected temperature. clove extract without $AgNO_3$ was kept as a control. Then the formed silver nanoparticles were characterized.

pH Optimization

The silver nitrate was added to the clove extract and the pH value of the reaction mixture was adjusted to 5, 7, and 9 and incubated for 24 hours. A control of clove extract without $AgNO_3$ was kept. Then the formed silver nanoparticles were characterized.

Characterization of Clove-AgNPs

The first insight of silver ions reduction to silver nanoparticles is the colour change of the reaction mixture from light brown to dark brown. Biosynthesis of Clove-AgNPs was confirmed by measuring the wavelength of the biosynthesized nanoparticles by the UV-Vis spectrum. Clove-AgNPs showed a scanning range of 300-900. Atomic Force Microscopy (AFM) was used to measure the diameter and surface granularity of the biosynthesized clove-AgNPs. A thin film of filtered prepared Clove-AgNPs was placed on a glass slide and allowed to dry at 25°C in dark conditions. Then the prepared slide was subjected to AFM analysis.

Testing the antibacterial activity of biosynthesized clove-AgNPs

The antimicrobial activity of clove-AgNPs was tested using the agar well diffusion method against hypermucoviscous *K. pneumoniae* (Logeswari *et al*., 2015). An 18-hour bacterial culture broth was inoculated homogenously on Mueller-hinton agar (Himedia-India). Then wells were done using a sterile gel borrer. The wells were loaded with the synthesized nanoparticles. After the plates had been incubated at 37°C for 24 hours, the inhibition zones around each well were measured.

Using the conventional broth dilution method, the Minimum Inhibitory Concentration (MIC) of clove-AgNPs was calculated following the CLSI standard. Using successive dilutions with concentrations from 8 $\mu g.mL^{-1}$ to 500 $\mu g.mL^{-1}$ and adjusted bacterial concentration, the

MIC in Muller Hinton broth (1×10^8 CFU.mL^{-1}, 0.5 McFarland standard) of hkp isolates. The time and temperature of incubation were 24 hours and 37°C respectively. Inoculated medium without clove-AgNPs was the positive control, whereas sterile broth medium was the negative control. The visible turbidity of the tubes after incubation revealed the MIC (Gurunathan *et al*., 2014).

The anti-biofilm formation efficacy of clove-AgNPs, was examined using the protocol described by (Barapatre *et al*., 2016; Mohanta *et al*., 2020). The anti-biofilm activity was tested by using serial two-fold dilutions with concentrations ranging from 8 µg.mL^{-1} to 500 µg.mL^{-1}. The test was performed using a 96 well microtiter plate. Overnight culture of hkp was cultured in TSB which was diluted to 1:100 (TSB + 1% w/v glucose). Each well of the microtiter plate was loaded with 100µl of medium and 100µl of colve-AgNPs; while the control well was loaded with 100µl of medium + 100µl sterile D.W. Each concentration for the clove-AgNPs, that evaluated triplicate. The plate was then incubated at 37ºC for 24 hours. To get rid of planktonic bacteria, the wells were once again cleansed with sterile D.W. After that, each well received 0.1% (w/v) crystal violet solution, and the plate was left to stain for 10 min. at room temperature. Placing the plate in a water dish allowed the crystal violet solution to be washed off. After wiping off any remaining liquid using paper towels, the dish was permitted to dry naturally. The dye was solubilized by treating the stained wells for 15 min. at room temperature with 33% (v/v) acetic acid. The optical density of the bacterial suspension in each well was assessed at 630 nm using a micro ELISA auto reader after being thoroughly mixed. The ability to form biofilm was estimated according to the O.D of the control in a special equation (Table 1).

Results & Discussion

Isolation and identification

Out of 200 food samples, 103 bacterial isolates were grown on MacConkey agar: 54 (52%) non-lactose fermenters with 49 (48%) lactose fermenters (Fig. 2). The small colonies of lactose fermneters were neglected, while large mucoid lactose fermenters were suspected to be *Klebsiella* (Fig. 3). Vitek system was used to confirm the identification to species level and the results revealed that the isolated bacteria is belonging to the species *Klebsiella pneumoniae.* Out of 200 samples resulted in the isolation of 28 *K. pneumoniae* isolates. These isolated foodborne *K. pneumoniae* were from different food sources: 10 (35.71%) from minced meat samples, 8 (28.57%) from chicken samples, 6 (21.42%) from vegetables, and 4(14.28%) isolated from cheese (Fig. 4). The findings of this investigation revealed that 28 *K. pneumoniae* isolates (14%) were isolated from 200 samples. These isolates of *K. pneumoniae* from food came from a variety of sources, including 10 (35.71%) from minced meat samples, 8 (28.57%) from chicken samples, 6 (21.42%) from vegetables, and 4 (14.28%) from cheese.

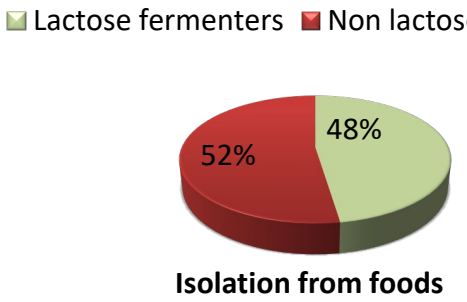

Fig. (2): Initial results of foodborne bacterial isolation.

Fig. (3): MacConkey agar cultured with food origin hypermucoviscous *Klebsiella pneumoniae*, large mucoid convex lactose fermenter after incubation at 37°C for 18 hours aerobically.

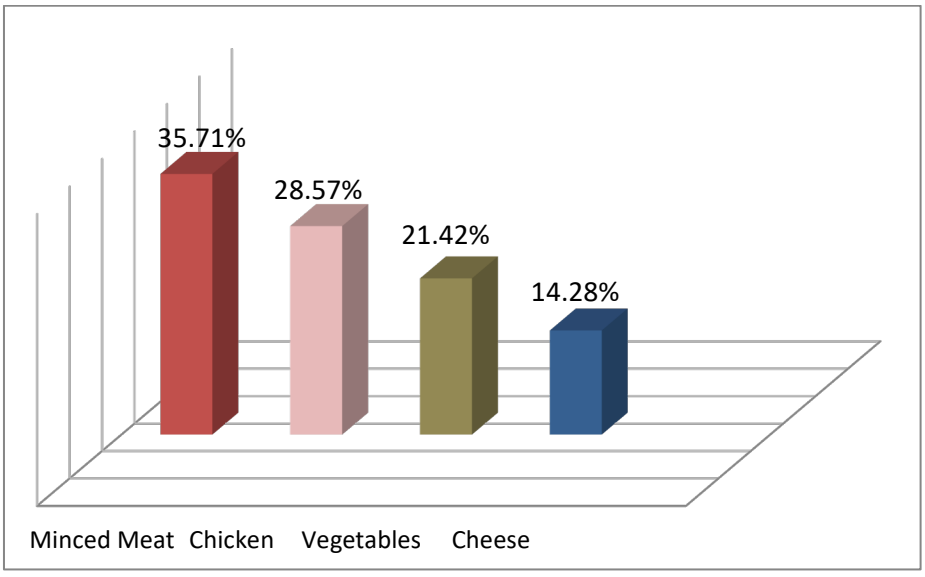

Fig. (4): The isolation percentage of *K. pneumoniae* according to each food source.

Junaid *et al.* (2022) conducted that involved the collection and handling of over 260 uncooked food products, including vegetables and fruits, meat, and milk specimens, for the isolation of *K. pneumoniae*. They observed that the occurrence of *K. pneumoniae* was significant (37%), with unpasteurized milk specimens being the most contaminated, followed by 23 % in fruits, 22% in meat, and 18 % in

vegetables. A similar study in China, looked at the frequency of *K. pneumoniae* isolates in uncooked and prepared food samples (Guo *et al*., 2016). The frequency of *K. pneumoniae* in Chinese cuisine was determined by a 9.9% incidence rate. They also observed that chicken was more contaminated than minced beef, mutton, and seafood, which contradicts our findings (Guo *et al*., 2016). The disparity in results might be attributed to differences in sample size, research region, and study sample source.

String test results showed that 16/28 (57.14%) *K. pneumoniae* isolates were hypermucoviscous. These isolates developed a continuous robe (5-11 mm) between the loop and the agar plate when the loop pulled outward Petri dish (Fig. 5).

Fig. (5): Positive result of String test (11 millimeter mucous thread) for hypermucoviscous *Klebsiella pneumoniae*

The string test, a common technique for assessing hypermucoviscosity, it was used to analyze these isolates for the hypermucoviscosity phenotype (Xu *et al*., 2021). In the current investigation, String test results showed that 16/28 (57.14%) *K. pneumoniae* isolates were hypermucoviscous. A recent study showed that 42% (21/50) of Kp isolates were determined to be hmvKp since they produced a viscous string that was longer than 5 mm, demonstrating a positive string test result. Comparatively, 58% (29/50) of isolates were non-hmvKp (Osama *et al*., 2023). In the literature, the terms hypermucoviscous and hypervirulent are used interchangeably (Elken *et al*., 2022). The hypervirulent (hvKp) is a common superbug that causes invasive diseases in hospitals and communities, including pyogenic liver abscess, meningitis, and endophthalmitis (Zhu *et al*., 2021; Elken *et al*., 2022). In a previous study; the bacteremia generated by hmvKp resulted in a 35% death rate (Lee *et al*., 2006). Five patients with bacteremia died in a case series of 12 individuals with hkp (Decre *et al*., 2011). Notably, Asian nations such as Taiwan, China, South Korea, and Iran have a significant prevalence of hvKp-related pyogenic liver abscess (Lee *et al*., 2016; Osama *et al*., 2023). It is unclear, however, whether the rise of hvKp in Asia is related to genetic predisposition in these groups, environmental factors, or other unknown variables (Shon *et al*., 2013).

Suckling mice bioassay results showed that 11/16 (68.75%) bioassay results on suckling mice revealed that 11/16 (68.75%) of foodborne hypermucoviscous *K. pneumoniae* isolates were enterotoxin producers. Kaur *et al*. (1988) found similar finding. They demonstrated that enterotoxicity was detected in 61 of 100 *Klebsiella* isolates (61%), depending on a coagglutination assay using anti-LT4 coated staphylococci. Singh & Kulshreshtha, (1992) on the other hand, tested 12 *K. pneumoniae* isolates for enterotoxicity in animals using the vaso permeability factor test, the rat foot pads assessment, the latex agglutination examination, and the coagglutination test, only one isolate of *K. pneumoniae* isolated from prawns from

freshwater was shown to be enterotoxigenic. The biofilm formation ability of hkp isolates was tested by two assays Congo Red Agar and microtiter plate. The results revealed that all hkp isolates were biofilm producers with different thickness degrees (Fig. 6).

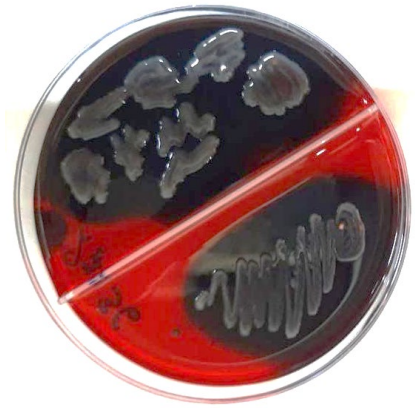

Fig. (6): Slime layer production by hypermucoviscous *Klebsiella pneumoniae* appeared as black colonies on Congo red agar after after 24 hours of incubation at 37°C in aerobic conditions.

In the current investigation, the potential of hkp isolates to produce biofilms was examined using two assays: congo red agar and microtiter plate. The findings demonstrated that all hkp isolates produced biofilms of varying thicknesses. Similarly, a previous study detected biofilm development in three *K. pneumoniae* isolates and discovered that each of them generated bio films (Saeed *et al*., 2013). Seifi *et al*. (2016) observed that biofilms formed on 93.6% of their *K. pneumoniae* isolates. Thirty-three percent of their isolates created fully established biofilms, 52.1% formed moderately biofilms, 8.5% generated poor bio films, and 6.4% produced no biofilms. Yang & Zhang (2008) studied the production of biofilms by *K. pneumoniae* strains obtained from urine, sputum, wound swabs, and blood. They observed that biofilms were generated by 62.5% of the strains, which is lower than our findings. This might be due to geographical and sample size differences. Rahal *et al*. (2021) found that the antibiotic resistant *K. pneumoniae* formed stronger biofilms than the sensitive isolates. The ability of nosocomial opportunistic bacteria such as *K. pneumoniae* to build biofilms on host-tissue surfaces is a critical stage in infection development. Biofilm formation affects the efficacy of antimicrobial therapy as well as the outcomes of subsequent infections (Borges *et al*., 2015).

Biosynthesis of Clove-AgNPs

The biological method which used in this study gave a positive result for the rapid, safe and cheap synthesis method of silver nanoparticles by clove aqueous extract. The biosynthesized Clove-AgNPs were characterized by colour change, UV, and AFM (Fig. 7).

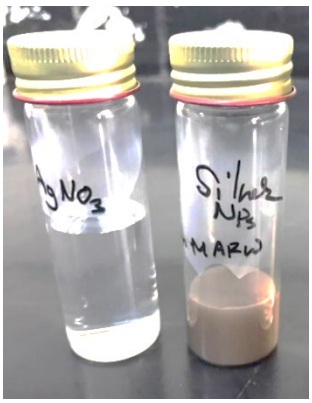

Fig. (7): Colour change incdicating the synthesis of silver nanoparticles in the reaction vessle.

Colour change to dark brown was the first insight of the nanoparticles formation in the reaction mixture (Kaur *et al*., 2013). Shareef *et al*. (2023) stated the colour change from green to brown as a first indicator for the green synthesis of AgNPs from okra plant. After being synthesized, silver nanoparticles were characterized to measure the average diameter by AFM technique and the results showed the formation of 14.12 nm nanoparticles (Table 2, Fig. 8).

Table (2): Granularity cumulation distribution report.

Average diameter	<=50% Diameter	<=10% Diameter	<=90% Diameter
14.12 nm	12.00 nm	6.00 nm	23.00 nm

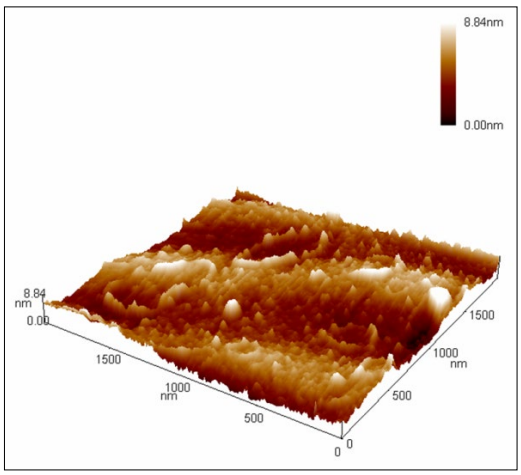

Fig. (8): Three dimensional image of Clove-AgNPs under the atomic force microscope.

The conditions for the Clove-AgNPs biosynthesis process were optimized in this study. Optimized conditions included several important parameters. The results revealed that the optimum conditions are: 1mM of $AgNO_3$ concentration, pH 7 , at 37 °C, and 24 hours (Table 3).

Table (3): Results of the optimization for the biosynthesis ofsilver nanoparticles.

$AgNO_3$ concentration	**Result**	**Temperature**	**Result**	**pH**	**Result**
1 mM	14.12 nm	4°C	negative	5	98 nm
2 mM	94 nm	37°C	14.12 nm	7	14.12 nm
3 mM	negative	80°C	80 nm	9	negative

The biosynthesis of Clove-AgNps was in optimized conditions that resulted in the formation of AgNPs with a diameter of 14.12 nm. Abbas & Faliyyah (2019) observed that the smallest nanoparticles were synthesized by bacterial isolate at pH 6 in 30°C. Tawfeeq *et al*. (2017) synthesized the AgNPs from banana peels using microwaves, this biological agent played the role of reduction, stabilizing and capping. The first indication of the AgNPs formation in the biosynthesis vessel is the colour change to a dark brown colour, The darkness of the brown colour in the biosynthesis vessel is dependent on the plant extract concentration (Almudhafar & Al-Hamdani, 2022).

Antimicrobial activity results

The biosynthesized clove-AgNPs exhibited antimicrobial activity against hypermucoviscous enterotoxigenic *K. pneumoniae,* both forms of life planktonic and

biofilm were inhibited. The minimum inhibitory concentration of the clove-AgNPs was detected as 62.5 µg.mL^{-1} and the subinhibitory concentration of 31.25 µg.mL^{-1} with the below concentrations was tested for antibiofilm activity.

The treatment of hypermucoviscous enterotoxigenic *K. pneumoniae* with concentration of 31.25 µg.mL^{-1} of Clove-AgNPs resulted in: 91% decrease in the biofilm formed, 83% for 15.62 µg.mL^{-1}, 76% for 7.81 µg.mL^{-1} and 3.90 µg.mL^{-1} resulted in 60% reduction in biofilm with significant difference ($p \leq 0.05$) as compared with control (Fig. 9).

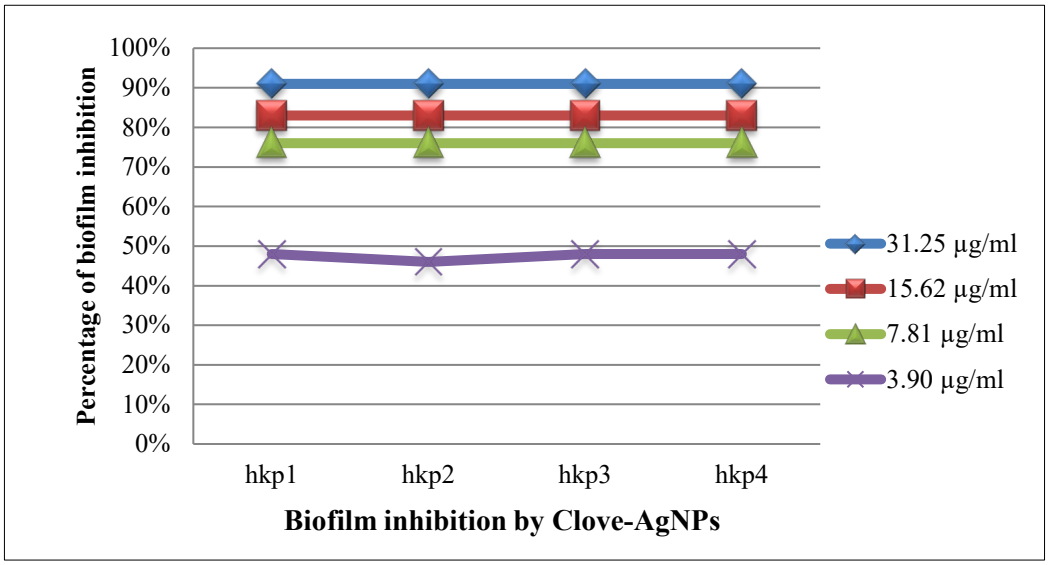

Fig. (9): Antibiofilm effect of sub-inhibitory concentrations of Clove-AgNPs against 4 isolates of hypermucoviscous *K. pneumoniae* (hkp) tested by microtiter plate assay

The biosynthesized Clove-AgNPs have antibacterial action against hypermucoviscous enterotoxigenic *K. pneumoniae*, inhibiting both planktonic and biofilm life forms. The minimum inhibitory concentration of clove-AgNPs was found to be 62.5 µg.mL^{-1}, while the subinhibitory concentration was found to be 31.25 µg.mL^{-1} when evaluated for antibiofilm activity at the examined doses. The treatment of hypermucoviscous enterotoxigenic *K. pneumoniae* with Clove-AgNPs at a concentration of 31.25 µg.mL^{-1} resulted in a 91% decrease in biofilm formation, 83% for 15.62 µg.mL^{-1}, 76% for 7.81 µg.mL^{-1}, and 60% reduction in biofilm as compared to the control. Khalil *et al.* (2020) studied 23 ESBL-producing strains (19.16%) (*E. coli*=17, *Klebsiella spp.*=6). They discovered that the nanoparticles of silver displayed a claiming antibacterial impact, with the MIC of AgNPs for ESBL-producing *E. coli* getting 0.31 mg.mL^{-1} and 0.62 mg.mL^{-1} for ESBL-producing *Klebsiella spp.*, respectively, and the MBC being 0.15 mg.mL^{-1} and 0.3 mg.mL^{-1}, respectively. The AgNPs have the ability to enter biofilms, which provides a practical method to inhibit biofilm formation (Saleh, 2020). Local studies showed that the sub-inhibitory concentrations of nanoparticles had an antibiofilm activity against Gram-negative bacteria. (Al-Khafaji, 2017, Al-Shaabani *et al.*, 2020).

The powerful antibacterial capabilities of silver against a variety of bacterial species, was the cause behind using it as an antibacterial agent for a very long time. Most

of the time, nanoparticles are preferred due to the various characteristics of the NPs, over the fundamental Ag-based materials. In the last decade, the science of nanobiotechnology has faced a significant challenge with the biosynthesis of AgNPs. This noble metallic nanoparticle has had a considerable impact on the biological, food processing, environmental, agricultural, and industrial fields due to its exceptional biophysical characteristics and increased biocompatibility. The disadvantages of conventional physical and chemical synthesis techniques have been solved by the widespread use of medicinal plant extracts as reducing and stabilizing agents in the green synthesis of AgNPs (Al-Khafaji *et al*., 2017). The biomolecule-encapsulated AgNPs are produced by reducing silver ions (Ag+) with phytochemicals found in plant extracts (Some *et al*., 2022).

The AgNPs are a promising bactericidal agent with enriched antibacterial action due to their reduced size and structure. Additionally, because the AgNPs have a large surface area, they can lend antibacterial effects to a large region of their host material (Ansari *et al*., 2019). As compared to other nanoparticles, AgNPs are more stable than copper and gold (Ibrhim, 2017). Because the AgNPs have a larger surface area than other salts, which results in better contact microbes, they exhibit effective bactericidal capabilities. It has been noted that when AgNPs reach the bacterial cell membrane, a low sub-atomic weight is created inside the cell. As a result, the bacteria aggregate together to protect the DNA from the AgNPs (Ahmad *et al*., 2020). Chemical synthesis, which has been utilized for decades, is the simplest and most practical method for the quick production of AgNPs. However, the chemical method of synthesizing AgNPs calls for the employment of various hazardous chemicals. Nanoparticles can be prepared in different ways may be a bio-physical method as using cold plasma with a biological agent (Mazhir *et al*., 2019). Another way that may be adopted is the microwave, Al-Ogaidi (2017) synthesized the silver nanoparticles by subjecting the silver nitrate solution with glucose to these waves.

It has been proposed to generate metal nanoparticles using microbes and plants, among other biological processes. Plant-based nanoparticle production is intriguing since it requires less effort than storing and growing microbes. Plant metabolites are useful in the production of AgNPs since they are natural and frequently have minimal toxicity to live cells (Mittal *et al*., 2013). There have been several articles on plant synthesis of AgNPs. These studies demonstrated how efficiently plant metabolites may create functional AgNPs. According to Dauthal & Mukhopadhyay (2016), the plant′s secondary metabolic products, which also include flavonoids, phenolic substances, glycosides, and sterols, are responsible for converting silver ions to their elemental form.

Because the plants vary in the content and amount of secondary metabolites, a wide range of plant types must be studied for their capability and usefulness in biosynthesizing metal-NPs. The use of phytomolecules in the manufacture of AgNPs may offer a solution to the problems caused by hazardous chemical reagents. Biosynthesis provides various advantages over traditional physical and chemical methods, including environmental concerns, economics, and ease of scaling for large-scale manufacturing. Furthermore, high temperatures, pressures, energy, or hazardous substances are not required in the production of nanoparticles (Ahmed *et al*., 2016). Additionally, the significance of developing

new techniques for producing AgNPs lies in their potent utilization as broad-spectrum antimicrobial and anticancer agents (Venugopal *et al*., 2017). An elevated resistance by the foodborne bacterial isolates. This may be resulted from the dual use of antibiotics in the veterinary field as growth promotion agents and for prophylaxis (Rodríguez-Félix *et al*., 2022). Abdul-Karim & Hussein (2022) stated that the application of green nanotechnology increased the plants resistant to pathogens.

Conclusion

According to the obtained data, the prevalence of hypermucoviscous *Klebsiella pneumoniae* in food samples is high, with a strong ability to produce biofilm. The biosynthesized clove-AgNps by sustainable method showed potential antimicrobial activity against enterotoxigenic hypermucoviscous *K. pneumoniae*.

Limitation of the study

The most important limitation of this study is that the biosynthesized silver nanoparticles (Clove-AgNps) were used to inhibit the growth of hypermucoviscous *K. pneumoniae* of food origin. While the mentioned studies have been used the biosynthesized AgNps to inhibit classical *K. pneumoniae* not hkp. In addition to that no previous study tried to eradicate the foodborne hypermucoviscous *K. pneumoniae*; they just focused on the inhibition of clinical isolates only.

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Contributions of authours

M.H.A, study design, carried out the experimental work, acquisition of data, proposal writing and drafting and revising the manuscript and submitted the final manuscript.

R.H.M, study design, planned methodology, read, revised and approved the manuscript.

A.M.A, study conception, analysis of data and drafting the manuscript.

ORCID

M.H.A.: https://orcid.org/0000-0002-2042-6231

R.H.M.: https://orcid.org/0000-0002-4007-5774

A.M.A.: https://orcid.org/0000-0002-8287-2579

Conflicts of interest

The authors declare that there are no conflicts of interest.

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التصنيع الحيوي لدقائق الفضة النانوية ذات فاعلية مضادة للبكتريا *Klebsiella pneumoniae* المحمولة بالغذاء فائقة اللزوجة المنتجة للسموم المعوية باستخدام المضاف الغذائي

مروى حميد مطشر الخفاجي*[1] ورغدان هاشم محسن [2] وايوب مرتضى الشيخ فخري [3]

[1*] قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

[2]قسم الانتاج الحيواني، كلية الزراعة، جامعة البصرة، البصرة، العراق

[3]الجامعة التقنية الوسطى، مؤسسة بلد التقنية، بغداد، العراق

المستخلص: هدفت هذه الدراسة إلى اعتماد طريقة صديقة للبيئة لتصنيع الدقائق النانوية ذات النشاط الفعال المضاد للبكتريا والأغشية الحيوية. عزلت بكتريا *Klebsiella pneumoniae* فائقة اللزوجة من عينات الاغذية وتم تشخيصها باستخدام الاختبارات الكيموحيوية ونظام فايتك. تم الاعتماد على اختبار المطاطية لتشخيص العزلات شديدة اللزوجة. تم الكشف عن السمية المعوية لعزلات *K. pneumoniae* فائقة اللزوجة المحمولة بالغذاء باستخدام الطريقة الحيوية للفئران الرضيعة. تم اختبار قابلية تكوين الأغشية الحياتية لجميع العزلات البكتيرية باستخدام طريقتي وسط احمر الكونغو واطباق المعايرة الدقيقة. تم استخدام المستخلص المائي للقرنفل *Syzygium aromaticum* للتصنيع الحيوي لدقائق الفضة النانوية في الظروف المثلى. تم تشخيص دقائق الفضة النانوية المصنعة حيويا من القرنفل (Clove-AgNps) بعدة تقنيات، وتم تحديد نشاطها المضاد للميكروبات والمضاد للأغشية الحياتية. أظهرت نتائج هذه الدراسة عزل 28 عزلة من بكتيريا *K. pneumoniae* من 200 عينة غذائية. أظهرت نتائج اختبار اللزوجة أن 28/16 (57.14٪) من العزلات كانت شديدة اللزوجة. وتبين ان أحد عشر عزلة من هذه العزلات (68.75%) كانت ذات سمية معوية. إن استخدام المستخلص المائي للقرنفل كعامل حيوي كان ناجحا في التصنيع الحيوي لدقائق الفضة النانوية بمعدل قطر 14.12 ملم بعد قياسها بمجهر القوة الذرية. كانت الظروف المثلى للتصنيع هي تركيز 1 ملي مولار من نترات الفضة ودرجة الحرارة 37 مئوية وقيمة الاس الهيدروجيني 7. تم تحديد التركيز المثبط الادنى من الدقائق النانوية المصنعة حيويا في هذه الدراسة ب62.5 ميكروغرام/مل. خفض التركيز تحت المثبط الادنى (31.25 ميكروغرام/مل) من الدقائق النانوية المصنعة حيويا نسبة 91% من الغشاء الحيوي المتكون ويمكن الاستنتاج أن استخدام *S. aromaticum* هو وسيلة فعالة صديقة للبيئة للتصنيع الحيوي لـ AgNPs ذات نشاط ممتاز مضاد للبكتيريا ومضاد للأغشية الحياتية ضد البكتريا فائقة اللزوجة المنتجة السموم المعوية.

الكلمات المفتاحية: مواد مضادة للميكروبات، غشاء حياتي، قرنفل، تسمم غذائي.