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In vitro Anti-biofilm Activity of Probiotic-mediated Biosynthesized Silver Nanoparticles

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Abstract

Background: Seventy samples were collected from the feces of healthy newborn babies aged (2-4) weeks and from the arrivals to vaccination centers feed by breast milk in Wasit province, Iraq. Babies were assessed clinically, placed directly in the sterile liquid MRS medium in test tubes and transferred to the laboratory to complete the isolation processes. **Methods:** Diagnosis of lactic acid bacteria isolates included: catalase test, gelatin liquefaction test and carbohydrate fermentation test for diagnosis of anaerobic bacteria in order to contain the number of confirmatory biochemical tests. Characteristics of isolates were compared with what exists in the antagonism activity assay against *Klebsiella pneumonia*e, determination of minimum inhibitory concentration, determination of minimum bacteriocidal concentration and detection of biofilm production using microtiter plate method was done. **Results:** By tissue microtiter plate method indicated that 1(3.3%) were strong for biofilms formation, while 6(20%) were moderate and 23 (76.7%) were demonstrated as a weak biofilm formation, (0%) were reported as nonbiofilm producing isolates. Microtiter plate method Antibiofilm effect of silver nanoparticles on 30 isolates of *K.pneumoniae* showed that the biofilm inhibition was tested, in which mean of control (biofilm formation without silver nanoparticles) was 0.21 compared to 0.07 using Bn1, while it was 0.21 compared to 0.08 as a results for antibiofilm using sub-MIC, (p value= 0.001, 0.004) respectively. **Conclusion:** Antibiofilm effect of silver nanoparticles on *K.pneumoniae* isolates of *Bifidobacterium* isolates showed that the biofilm inhibition has a significant effect.

Key words: Silver Nanoparticles, Biofilm and Probiotic

Introduction

Today, the term "probiotic" refers to "live microorganisms which when administered in adequate amounts, confer a beneficial physiological effect on the host," according to the Food and Agriculture Organization and World Health Organization (1, 2). The normal flora of the human gastrointestinal tract contains many diverse populations of bacteria which play an essential role in the development and well-being of the host. In the intestinal microflora exerts a protective role against pathogens (3, 4). Most commonly used probiotic supplements contain the species of Lactobacillus and Bifidobacterium and they are the part of normal human intestinal microbiota

(5, 6). The antagonism of lactic acid bacteria is exerted by competition for nutrients and for physical location, but also through the production of antimicrobial substances (7). These compounds are able to inhibit the growth of harmful microorganisms and the most important LAB are lactobacillus spp and Bifidobacterium spp (1, 8).

As a global public health issue, antimicrobial resistance (AMR) is a growing problem that occurs when microorganisms such as bacteria, viruses, fungi, and parasites become resistant to antimicrobial drugs that have previously served as effective treatments for infections (9). When bacteria become resistant to antibiotics, they can spread infections which are difficult to treat, which can result in prolonged illness, disability and even death as a result (10). Multi-drug resistance (MDR) is a form of AMR in which microorganisms become resistant to multiple drugs, making it more difficult to treat infections (11). This can happen when antibiotics are overused or misused, as well as when there is poor infection prevention and control in healthcare settings (12).

Recently, a lot of attention has been paid in finding ways to produce and use the nanomaterials, and the interest is growing every day. In terms of manufacturing nanoparticles, one of the methods to be considered is the bio-approach (13). Using microorganisms for synthesis of nanoparticles, for example, is referred to as nanoparticles synthesis by biological means. Nanoparticles are produced both by living and dead microorganisms, contribute greatly to nanoparticle production (14). An array of microorganisms such as *k. pneumoniae, E. coli*, *S. aureus*, *P. aeruginosa* and *S. typhus* may be susceptible to AgNPs as antimicrobial agents (15). Hence, as an emerging method for discovering antibacterial involves using green synthesized nanoparticles to target bacterial biofilm and QS. Utilization of silver nanoparticles as an alternative antimicrobial agent has been suggested (16). Nano-scaled materials appear to exhibit better biological effects than their bulk counterparts because their chemical and physical properties are different at this scale, which is mainly why the nano-scaled materials shown improved biological activity (17).

Probiotic bacterium that has gained considerable attention in the field of medicine due to its potential health benefits. As a naturally occurring bacterium in the human gastrointestinal tract (18). The synthesized AgNPs-LR were investigated for their broad-spectrum effect on inhibiting the virulence factors of resistance bacterial species (19, 20).

Materials and Methods

Samples Collection

Seventy samples were collected from the feces of healthy newborn babies at the age of (2-4) weeks, samples were collected from Wasit province, from 2022 to February 2023. The patients were first assessed clinically by the doctors in the hospital and then referred for sample collection (4).

Diagnosis of Lactic Acid Bacteria Isolates

The isolates underwent biochemical tests that included: Catalase test, Gelatin liquefaction test and Carbohydrate fermentation test for diagnosis of anaerobic bacteria in order to contain the number of confirmatory biochemical tests. Characteristics of isolates were compared with what exists in the (1).

Klebsiella pneumonia isolates

Thirty isolates of *K. pneumoniae* were obtained from (21).

Antagonism Activity Assay

Cell-Free Extract (CFE) Preparation

Cell-free extract of all lactic acid bacteria used in this study was prepared according to (22) as follows: Bifidobacterium spp. include (6) strains which were inoculated separately broth as 2 % of broth volume and incubated under anaerobic condition at 37°C for 72 h. The culture was then centrifuged at 5000 rpm for 30 min. The Supernatants were sterilized by filtration through (0.22µm) membranes (Millipore filter paper-Swinnex-25).

Determination of Minimum Inhibitory Concentration

The MIC of CFEs for the test strains was determined according to (23). One isolate of *Bifidobacterium* fresh culture was inoculated in 10 ml nutrient broth containing filter concentrates of the culture supernatant (50,100,150,200) µl and incubated aerobically at 37˚C for 24 h for MIC determination (6).

Determination of Minimum Bacteriocidal Concentration

After the serial dilution for every treatment was done, then the bactericidal activity of CFE (*Bifidobacterium*) was determined by plating (0.1 ml) for each treatment into Mueller Hinton sterilized petri dishes and then incubated at 37˚C for 24 h, after incubation, results were recorded and compared with the control treatment (0% of CFE) (1, 6).

Detection of Biofilm production

Microtiter plate method

According to (21), biofilm formation test was detected by microtiter plate method.

Synthesis silver nanoparticles by biological method

Preparation of stock solution of AgNo3

Preparation of stock solution of AgNo3 by dissolving 0.085g of solid AgNo3 with 10ml of distilled water and stirred for 30 min by a magnetic stirrer and kept until used for preparation concentration 10mM (24).

Preparation of bacterial supernatant

To produce the biomass for biosynthesis, *Bifidobacterium* is culture in nutrient broth or LB medium incubated rotary shaker (200 rpm) for 37°C at 24 hr then the supernatant is collected by centrifugation at 10,000 rpm for 10 min. The supernatant was used for synthesis of AgNPs (25).

Synthesis of AgNPs

Ten milliliters (10ml) of supernatant is mixed with 10ml of AgNO3 solution to form 10mM concentration and other tube without silver nitrate serve as control supernatant without silver nitrate, pH of reaction was 9.0 and the prepared solution were incubated at 37°C for 48 hr. All solutions are kept in dark to avoid any reaction during testing after incubation the solution changed from yellow to brown solution the AgNPs are collected by centrifugation at 10,000rpm, for 5 min twice (24).

Characterization nanoparticles

Identification of silver nanoparticle (AgNPs) in Nano-center (Technology University) by using:.

Scanning Electron Microscopy (SEM)

A scanning electron microscope has resolution 3nm at 30kV take AgNPs images. The assembly involved with a computer software programming to analyze the mean size of the particles in sample (1).

X-Ray Diffraction (XRD)

According to Ramalingam *et al*., (2014) , "X-ray diffraction ,40 KV volume and with current (20Ma) , is used to identify the crystalline phases and to evaluation the crystalline size and the XRD patterns recorded with 2s in the range of (10r -60r) by step scanning, employing Cu tube with wavelength of Cu 1.54 A".

Fourier Transform Infra-red Spectroscopy (FTIR)

FTIR analysis of the AgNPs carried out on FTIR 8400S, (SHIMADZU-FTIR) spectrophotometer in the range 400–4000 cm^{-1} (26).

Effect of silver nanoparticles on biofilm production

Microtiter plate method

According to (27), effect of silver nanoparticles on biofilm formation by microtiter plate method.

The inhibition rate (%) was read as following

Inhibition rate (%) = OD of control - OD of treated / (OD of control) × 100

The microtiter plate antibiofilm assay estimations the percentage of bacterial biofilm reduction. In comparative to the control wells, which were fixed at 100% to indicate the absence of silver nanoparticles. In compare, negative percentage results indicate no inhibition activity of AgNPs on biofilm association.

Results and Discussion

Biofilm Production Assay

The results of biofilm formation to *Klebsiella pneumoniae* isolate (30 isolates) obtained from (21). by tissue microtiter plate method indicated that 1(3.3%) were strong for biofilms formation, while 6(20%) were moderate and 23(76.7%) were demonstrated as a weak biofilm formation, (0%) were reported as nonbiofilm producing isolates, table (1) and figure (1).

Table (1) Biofilm formation result of *Klebsiella pneumoniae* using microtiter plate method

Biofilm formation	OD630 limits number of isolates	Number of isolates	percentage
Non-adherent	<0.06-0.12	0	0%
weak	0.12-0.24	23	76.7%
moderate	0.132-0.264	6	20%
strong	≥0.24	1	3.3%

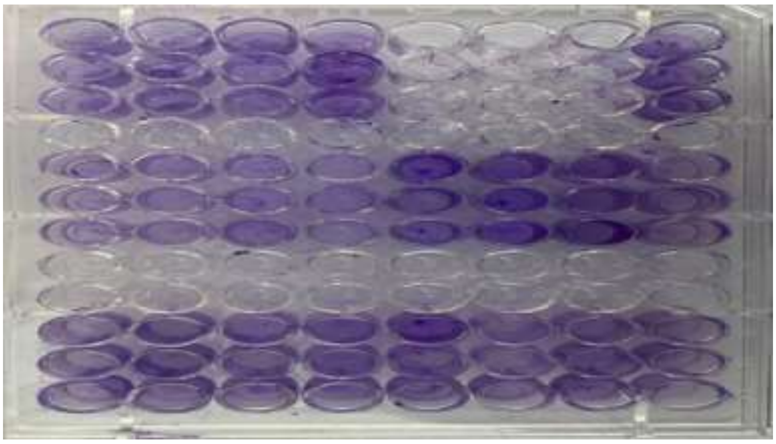

Figure (1). Biofilm production in *Klebsiella pneumonia*

Biosynthesis of silver nanoparticles

Using supernatant of *Bifidobacterium* in Synthesis of Silver nanoparticle

Nanoparticle synthesis in the used medium has been noticed by color change of *Bifidobacterium* supernatant with an aqueous solutions of silver nitrate (concentration of 10 mM) from yellow to brown after forty-eight hours after incubation.

Characterization of synthesized silver nanoparticles

Scanning Electron Microscopy (SEM)

Figure (2) illustrates the morphological characteristics of produced silver nanoparticles examined by SEM apparatus. Ag-NPs had reduced aggregation , and 35 nm was the average size (28).

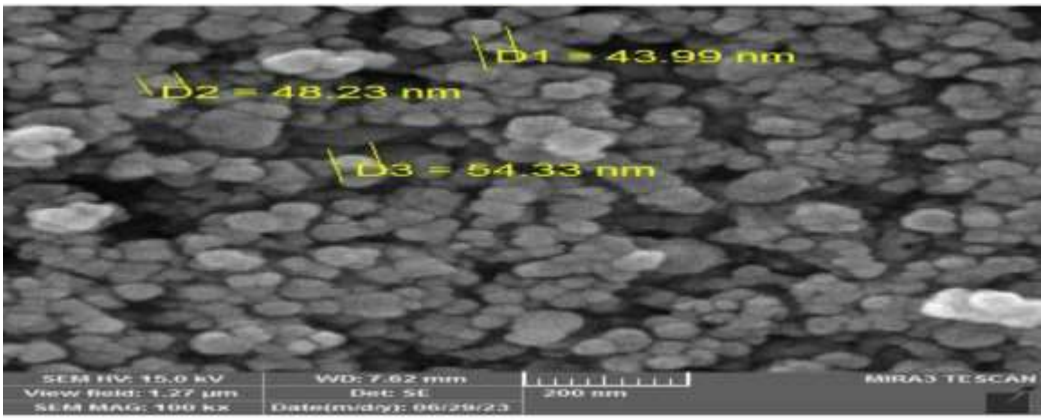

Figure (2): SEM image of Ag-NPs synthesized by *Bifidobacterium*

X-ray Diffraction (XRD)

Crystal structure of AgNPs was examined by XRD the, figure (3) exhibited one high peak is visible in the XRD spectrum of produced silver nanoparticles at 2ø (32.5°) at which corresponds to the (101) plane of conventional XRD data of nano silver Crystals (29).

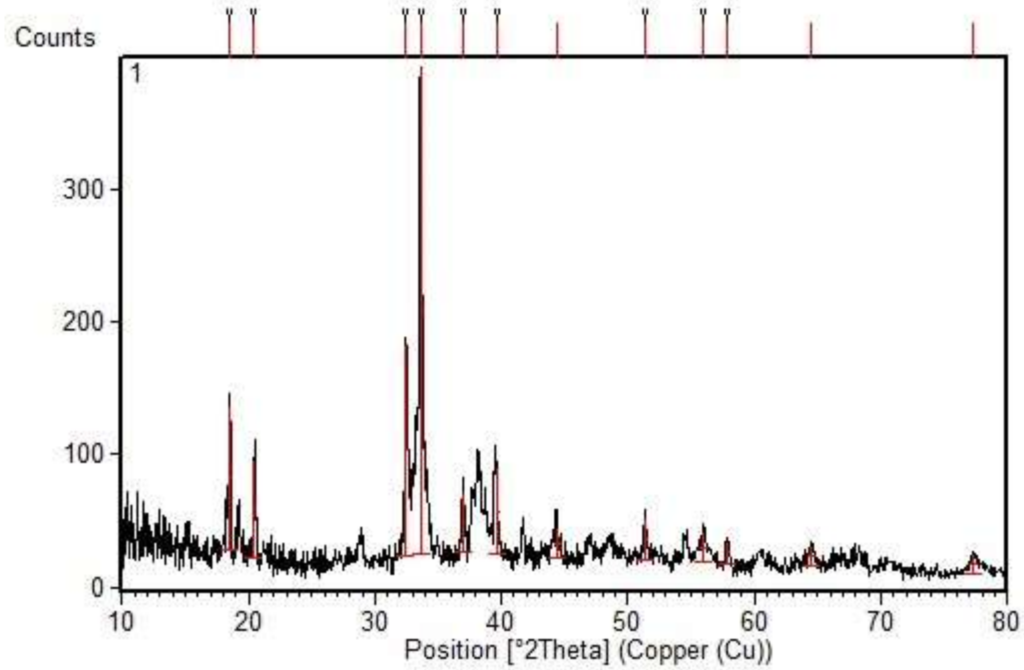

Figure (3): XRD range of synthesize Ag-NPs

Fourier Transform-Infrared spectroscopy (FT-IR)

FT-IR analysis of AgNPs synthesized by *Bifidobacterium* noted in the range (450 -4000) cm-1 as shown in table (2) and figure (4) ,it is useful for determining the chemical composition of reactants involved in the synthesis and coating of AgNPs.

Table (2) FT-IR analysis of AgNPs synthesized

Spectrum Name	Peak Number	X (cm-1)	Y (%T)
	1	3780.20	15.86
B	2	3426.15	13.81
	3	1383.03	11.27

	4	1101.77	11.42

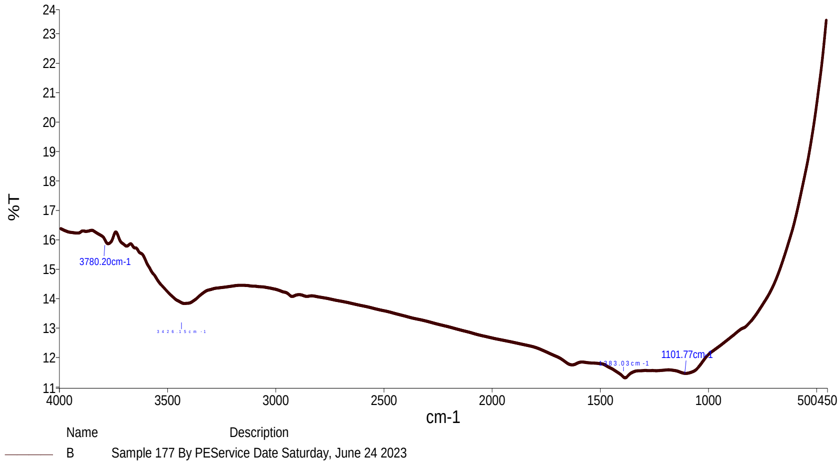

Figure (4) : FTIR analysis of silver nanoparticles

Determination MIC and Sub-MIC of Silver nanoparticles

In this study, Ag-NPs were tested against 30 isolates of *K .pneumoniae* by using a Resazurin-mediated microtiter plate and well diffusion method, the color change of the Resazurin indicator was used to visually reflect the inhibitory action. The results have shown that silver nanoparticles has inhibitory action (MIC) against *K .pneumoniae* with concentration 10Mm and sub-MIC with concentration 5mM . Determination of MIC is important for bacteria to determine the lowest concentration of AgNPs that is necessary for inhibition of visible bacterial growth after incubation period at 37°C for18-24 hr (30).

Testing the effect of silver nanoparticles on biofilm formation

Microtiter plate method

Antibiofilm effect of silver nanoparticles on 30 isolates of *K.pneumoniae* showed that the biofilm inhibition was tested, outcomes were illustrated in table (3) and (4), in which mean of control (biofilm formation without silver nanoparticles) was 0.21 compared to 0.07 using Bn1, while it was 0.21 compared to 0.08 as a results for antibiofilm using sub-MIC, (p value= 0.001, 0.004) respectively.

Table (3) and (4) Determination of biofilm and anti-biofilm in *Klebsiella pneumoniae* using silver nanoparticle

Sub-MIC	Biofilm in *K. pneumonia*	Anti-biofilm using sub-MIC	Mean
		Bn1	
16	0.35	0.18	0.26
64	0.21	0.05	0.13
8	0.22	0.11	0.11
16	0.21	0.09	0.15
4	0.20	0.03	0.11
16	0.22	0.09	0.15
64	0.10	0.01	0.05
64	0.21	0.02	0.11
Mean	0.21	0.07	0.13
P value	**0.001**		

Sub-MIC	Biofilm in *K. pneumonia*	Anti-biofilm using sub-MIC	Mean

		Bn2	
64	0.35	0.20	0.27
128	0.21	0.10	0.15
32	0.22	0.18	0.20
32	0.21	0.15	0.18
16	0.20	0.05	0.12
64	0.22	0.10	0.16
128	0.10	0.04	0.07
128	0.21	0.06	0.13
Mean	0.21	0.08	0.16
P value	**0.004**		

Discussion

In a previous study (31) it has been proposed the using of medium contain MRS added to l-vancomycin, bromocresol purple and cysteine, this will provide optimum condition in isolation and counting probiotic. It was explored the probability to improving "LcS" select medium by (31) used all MRS component.

In biofilm formation, the structure and regulatory genes play important role that effect on colony aggregation by different mechanisms ,as well as alteration of synthesis of transcriptional factors and regulation of extracellular polysaccharide production (32). In agreement with some local studies, (33) indicated that 100% of clinical *K. pneumoniae* isolates were able to produce biofilm. Also, (34, 35), observed that of 30 *K. pneumoniae* isolates; 23 (46%) were weak-biofilm producers.

Findings are in line with those of several earlier research like that by (36) study the results show that the MTP method was more sensitive in detecting biofilm production compared to the CRA. (37) (38) and (39) studies showed that there was no relationship between the two methods, and the MTP method is the most sensitive and realistic.

Strong bands at (1101.77, 1383.03, 3426.15 and 3780.20 cm−1) were discovered by FT-IR analysis of Ag-NPs. The stretch for Ag-NPs discovered around 1101.77 cm-1, and the other band at (3780.20) corresponds to –OH- free, (1383.03) shows the H–C–H Asymmetric, while band at (3426.15) for O-H stretching corresponds to carboxylic acid, corresponds OH-bend resembling to phenolic compounds which can potentially affect the synthesis and stability of AgNPs, these results are in agreement with previous reports (40, 41). (42) illustrated how silver weakens biofilm formation, when AgNPs transported into the cell and interact with proteins and enzymes, which are essential for microbial development quorum sensing or adherences that resulted in the decline in biofilm action. AgNPs inhibited biofilm formation by blocking the formation of exopolysacharides also the silver nanoparticles move during the water channels used for nutrient moving and spread through exopolysacharides layer (43).

Results of (34) study has revealed that mutation occurred in the gene after treatment with AgNPs, which may lead to the effect of the phenotype because they lead to change in amino acids and then protein that transition mutation was repeated 2 time at adnine and guanine bases that change amino acid.

In current study antibiofilm effect of silver nanoparticles on 30 isolates of *K.pneumoniae* showed that the biofilm mean of control (biofilm formation without silver nanoparticles) was 0.07 compared to 0.008 using Bn1 as inhibitor, while it was 0.93 compared to 0.30 as a results for antibiofilm using sub-MIC, (p value= 0.004 and 0.001) in microtiter plate method. These outcomes were agreed with (44). Probiotics are the live micro-organisms having host beneficial effects by enhancing microbial balance in intestine, whereas, prebiotics are indigestible food components having beneficial effects by enhancing the activity and growth of one or more colonic bacteria (45).

According to (46, 47) studies the rate of biofilm formation increased, the uptake of silver nanoparticles would be significantly reduced. Bacterial growth was suppressed by more than 90% when nanoparticles were present at a given concentration. The organism is unable to develop biofilm when exopolysaccharide synthesis is halted.

Characteristic or synergist effect of AgNPs, the release of Ag+, and the mode of action of AgNPs and bacterial extracts against bacteria all contributed to the increased antibacterial activity of AgNPs. The results of bacterial growth Bacteriostatic, bactericidal, and inhibitory of bacteria have all been shown to be effective against bacteria. The bacteriostatic, bactericidal, and biofilm inhibitory properties of AgNPs are all dependent on the concentration of AgNPs, according to (48).

Present findings are consistent with (49, 50) found that using antibiotics like ampicillin and ceftriaxone in combination with silver nanoparticles increased the efficiency (percentage) of antibiotics like ampicillin and ceftriaxone against bacteria.

Synthesized and characterized a probiotic *Bacillus licheniformis* cell free extract (BLCFE) coated silver nanoparticles (BLCFE-AgNPs). These BLCFE-AgNPs were characterized by UV–visible spectrophotometer, XRD, EDX, FTIR, TEM and AFM. A strong surface plasmon resonance centered at 422 nm in UV–visible spectrum indicates the formation of AgNP, these results suggests that BLCFE-AgNps may be used for the control of biofilm forming bacterial populations in the biomedical field (51). (52) reported a different visible light irradiation effects on the formation of silver nanoparticles from silver nitrate using the culture supernatant of *Klebsiella pneumonia*.

Conclusion

(53) and (54) indicated that antibiotic resistance against present antibiotics is rising at an alarming rate with need for discovery of advanced methods to treat infections caused by resistant pathogens. Silver nanoparticles are known to exhibit satisfactory antibacterial and antibiofilm activity against different pathogens, the percentage biofilm inhibition was evaluated to be 64% for *K. pneumoniae* strain MF953600 and 86% for MF953599 at AgNP concentration of 100 μg/ml. AgNPs were evaluated to be minimally cytotoxic and safe at concentrations of 15-120 μg/ml. The data evaluated provided evidence of AgNPs being safe antibacterial and antibiofilm compounds against MDR *K. pneumonia*. Contrary to our results, one of the previous studies by (55) demonstrated an increase in biofilm biomass of *Pseudomonas aeruginosa* after treatment of cells with superparamagnetic iron oxide nanoparticles at a concentration of 0.2 mg/ml. This study suggested that iron nanoparticles could be used as a source of elemental iron by the cell that is why they observed an increase in biofilm biomass with corresponding increase in cell density.

Competition Interest

Authors of this research showed there is no conflicts of interest.

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Authors contribution

Hajar T. Mahdi: Material preparing, questionnaire designer, data collection, explaining of outcomes and writing on article.

Prof Dr. Zainab N. Al-Saadi: Article proposal designer, statistical analysis, editing and writing.