SAR Journal of Pathology and Microbiology

Abbreviated Key Title: *SAR J Pathol Microbiol*
Home page: https://sarpublication.com/journal/sarjpm/home
DOI: 10.36346/sarjpm.2023.v04i04.003

OPEN ACCESS

ISSN 2707-7756 (P)
ISSN 2709-6890 (O)

Original Research Article

Iron Oxide Nanoparticles as Anti-Virulence Factors of Gram-positive and Gram-negative Bacteria

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Article History: | Received: 05.07.2023 | Accepted: 07.08.2023 | Published: 11.08.2023 |

Abstract: ***Background*:** Due to their extensive use in medical therapy, iron oxide nanoparticles have recently attracted the attention of researchers in the field of increasing multi-resistance properties in bacterial pathogens. Because iron oxide nanoparticles have a high specific surface area, they can interact with bacterial surface structures and exhibit significant antibacterial activity. ***Objective*:** The current work, determined the effect of a novel anti-virulence factor agent which was created from iron oxide nanoparticles against selected gram-positive and gram-negative variant bacterial strains that were isolated and identified from elderly Iraqi patients with urinary tract infections. ***Methods*:** Seven bacterial strains (three gram-positive and four gram-negative) were tested for their (biofilm, adhesion, and hemolysis) activity using the quantitative test-tube method, glass-slide method, and by determining the hemolysis ability of bacterial isolates against previously prepared RBC, respectively. The highest virulence factors formation bacterial isolates were chosen to determine the effect of Iron-oxide nanoparticles. Previously prepared and characterized Iron-oxide nanoparticles were used as anti- (biofilm, adhesion, and hemolysis) by using specific Bacterial-Fe3O4 NPs complex with different concentrations. ***The Results***: All these bacteria expressed their virulence factors, the highest-level biofilm formation abilities were detected in *Proteus mirabilis* and *Staphylococcus aureus*, and the highest-level adhesion activity was observed in *Enterococcus faecalis* and *Pseudomonas* aeruginosa while the highest-level hemolysis activities on human RBC were determined in *Micrococcus luteus* and *E. coli*. The effects of (Fe_3O_4) nanoparticles against the highest virulence factors bacterial isolates shows an increases in the biofilm formation abilities of *S. aureus* and *P. mirabilis* as well as for standard bacterial strains the anti-biofilm formation ability of (Fe3 O4) NPs against gram-positive *S. aureus, Proteus mirabilis, and standard gram*-negative bacteria demonstrated an in-decrease biofilm formation ability of these bacteria effective at (5000 and 10000 µg/ml). The anti-adhesion ability of (Fe3 O4) NPs against gram-positive *Enterococcus faecalis*, and gram-negative *Pseudomonas aeruginosa* determined no effect on the adhesion abilities of gram-positive and negative bacteria atall concentrations (250, 400, 500, 750 and 1000µg/ml) Finally, the effect of different concentrations of iron oxide nanoparticles on the hemolysis ability of *Micrococcus luteus* and *Escherichia coli* on RBC was determined, the highest hemolysis inhibition level was estimated in 1000 µg/ml and the less inhibition in 500 µg/ml as compared with control.

Keywords: UTI, bacteria, virulence factor, iron oxide nanoparticle, Baghdad.

INTRODUCTION

Introduction In elderly persons, urinary tract infections (UTI) and asymptomatic bacteriuria are prevalent (Rowe and Juthani-Mehta *et al.*, 2013). UTIs are classified in a variety of ways depending on the site of infection, the reasons that cause infection, and the symptoms that assist clinicians to diagnose UTIs and treating patients (Awaness *et al.*, 2000). The most prevalent bacterial illness in elders is UTI. Furthermore, in older individuals, this disease is the most common cause of community-acquired bacteremia and sepsis. UTIs range in severity from a simple self-limiting disease to severe sepsis, in patients over the age of 70, both sexes acquire UTI, with a female-to-male ratio of 2:1, contrasted to the overwhelming UTI susceptibility

of females in younger populations, with a 50:1 ratio (Gharbi *et al.,* 2019; Shallcross *et al.,* 2020). Both Gram-negative and Gram-positive bacteria, as well as certain fungi, can cause UTIs. Uropathogenic *Escherichia coli* is the most common cause of both uncomplicated and complicated UTIs (UPEC). *Klebsiella pneumonia, Staphylococcus saprophyticus, Enterococcus faecalis, group B Streptococcus (GBS), Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida spp*. are the most common agents involved in simple UTIs. *Enterococcus spp., K. pneumonia, Candida spp., S. aureus, P. mirabilis, and P. aeruginosa* are the most prevalent causal agents causing complicated UTIs, with UPEC being the most common (Flores-Mireles *et al.,* 2015). Discovering virulence factors of pathogenic bacteria is a key to understanding the pathogenesis and the identification of targets for novel drugs and the design of new vaccines. CAUTIs (catheter-associated urinary tract infections) are one of the most prevalent illnesses acquired in hospitals (Cepas *et al.,* 2019). Bacteria frequently associated with CAUTI include uropathogenic *Escherichia coli (UPEC), Enterococcus spp., Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa,* etc. (Mandakhalikar *et al.,* 2018).

Most commensal and pathogenic bacteria that interact with eukaryotic hosts have sticky molecules on their surfaces that enable them to engage with host cell receptors or soluble macromolecules. Many harmful bacteria have overcome this problem by developing an antiphagocytic surface layer of polysaccharides and expressing their adhesins on polymeric structures that protrude from the cell surface (Kline *et al.,* 2009). In the last two (2015-2020) decades, the metal oxides nanoparticles (NPs) of the transition metals (TM), have been used in a very wide applications such as sensors, adsorbents, catalysts, conductors, magnetic, and superconductors (Ismail *et al.,* 2015). Iron oxide NPs is one of the TM oxides that have a special morphology and other properties in their nano-scale structure that enables it to be used in variant applications among them as an antimicrobial agent in treating and preventing infectious diseases in animal and human beings (Das *et al.,* 2020). The using of antimicrobial agents plays an important role in reducing the effect of infectious diseases and decreasing the percentage of death (Ali *et al.,* 2016). Many studies and research indicated that nanoparticles (NPs) can be used as antibacterial agents and showed that their influences may variant according to their structure have different influences on pathogenic bacteria (gram-negative and gram-positive) and can prevent them from developing the disease and limit its activity (Dadfar *et al.,* 2019, Zhou *et al.,* 2021).

MATERIAL AND METHODS

Bacteria isolate

Proteus mirabilis, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Micrococcus luteus, and E. coli these bacteria were kindly gifted by (Amin et al., 2021) and it was diagnosed biochemically and confirmed by Vitek.

Characterization and chemical synthesisof Iron oxide (Fe3O4) Nanoparticles were made by (Amin *et al.,* 2021).

Detection of bacterial virulence factors

Bacterial Biofilm formation assay (Test tube method)

Quantitative evaluation of bacterial isolates for biofilm formation was done according to the methods described by (Hasan *et al.,* 2014; Haney *et al.,* 2018) An overnight 10 µl of a stock bacterial culture with (O.D 620=0.01) inoculated into 1ml of pre-sterilized glass tubes containing sterile Brain heart infusion broth, incubation was made at 37oC for 2 days (48 hrs.). Thus, the formation of biofilm in all test tubes was monitored at the incubation interval. After the specified incubation period, the culture broths from the tubes were decanted and the adherents were rinsed three times with distilled water then tubes were patted dry on a paper towel. To each tube 1ml of 0.1% crystal violate solution was added to stain the adhered biomass and the tubes were incubated at room temperature for 30 minutes. After that the crystal violates dye was discarded and the tubes were again rinsed three times with distilled water and patted dry. The tubes were photographed to document the amount of biofilm that was adhered to the glass tube surface, then 1ml of 70% ethanol was added to each tube to release the bound crystal violate dye from the biofilm. Quantification was done by using a spectrophotometer O.D 595nm. Each bacterial isolate was evaluated in triplicate under the previous growing conditions.

Adhesion

Adhesion test for all bacterial isolates was carried out according to the method described by (Zeraik and Nitschke, 2012) as follows: Bacterial suspension 200µl culture was adjusted to 0.5 McFarland (108 /CFU) spread on 1Cm2 glass then incubation was done for 4 hours at 37oC.The slides were then washed 3 times with distilled water. In order to fix the adhered cells 200µl of methanol was flooded over each slide for 15 minutes, therefore the non-adherent cells were removed by distilled water. To the slides added 200µl of crystal violet (1% w/v) and leave for 15 minutes. The stain slides were washed with tap water, while the bounded stain was solubilized with 200 µl of 33% glacial acetic acid. The optical density of the solubilized stain was measured by an ELISA reader at 630 nm (Laloy *et al.,* 2014).

Hemolysis

Hemolysis is defined as the release of hemoglobin into plasma due to damage of erythrocytes membranes, which was determined by the method given by (Rozalski and Kotełko, 1987) (Kotelko *et al.,* 1983). To determine the hemolysis ability of bacterial isolates against previously prepared RBC, 0.2 ml of 18 hours

brain heart infusion broth culture was inoculated into 2ml of the same broth and o.2 ml of RBC 5% standardized human blood erythrocyte suspension. After 3 hours of incubation at 37oC, the mixture was centrifuged at 1500xg and the amount of hemoglobulin that was released in the supernatant was measured by spectrophotometer with O.D. 540 nm. The blank sample is composed of the previous constituents but at zero time (before incubation). Composition of control positive 2.2 ml of 1% Triton x 100 and 0.2 ml of 5% RBC (incubated for 3 hours). Control negative composed from Ringer solution (normal saline) 2.2 ml with 0.2ml of 5% RBC suspension (incubated for 3 hours). Blanks for each control positive and negative are composed of the same constituents for each without incubation. (At zero time). For each bacterial isolate, work was done in triplicate as well as for control positive and negative.

The effect of (Fe_3O_4) NPs against bacterial virulence factors

Preparation of (Fe_3O_4) nanoparticles against virulent factors

In all experiments concerning the use of iron oxide (Fe3O4) nanoparticles against bacterial virulence factors, preparation of nanoparticles was done as follows: (Fe_3O_4) nanoparticles 0.005 gram was dissolved in 2 ml of dimethyl sulfoxide (DMSO) to get a concentration of 10 mg/ml, then the solution was placed in an ice bath and exposed to ultrasound waves for 5 minutes using (Sonicator) until it became colloidal.

Preparation of bacterial isolates

In all experiments concerning the use of iron oxide (Fe3O4) nanoparticles against bacterial isolates' virulence factors, bacterial isolates were prepared as follows: Bacterial isolates were grown overnight with incubation at 37oC in 5ml brain heart infusion broth then bacterial growth adjusted to 0.5 McFarland Chapter Three Materials and methods 53 turbidities equivalent to (1.5x108) Colony Forming Unite (CFU), which represent stock solution.

Anti-biofilm formation assay

The effect of iron oxide (Fe3O4) NPs against bacterial biofilm formation was conducted using (Test tube method) and according to the procedure described by (Hassani *et al.*, 2015) as follows: A serial dilutions of previously prepared colloidal iron oxide NPs (250, 500, 750,1000,5000, and 10000 µg/ml) with bacterial growth stock culture and sterilized brain heart infusion broth were prepared in test tubes and incubated for 24 h at 37 ºC, the contents of tubes were removed and washed 4 times with PBS (pH = 7) to remove free bacteria. Bacterial biofilms were fixed with 95% ethanol and stained with 0.1% (w/v) crystal violet after that rinsed off 5 times with D.W. to remove excess stain and kept to dry. 225 µl of 33% glacial acetic acid was added to each tube and read after 15 minutes by spectrophotometer reader at O.D 590 nm. The absorbance considered the value of bacterial adhesion on the surface of nanoparticles and biofilm formation. The average of triplicate reads of each concentration was calculated. Control negative preparation for each bacterial isolate was made as previously described under the same conditions and additions with the exception that bacterial suspension was replaced with distilled water.

Anti-adhesion assay

The effect of iron oxide (Fe3O4) NPs against bacterial adhesion was conducted using the same method described in (2.1.2) with little modification as follows: A serial dilutions of previously prepared colloidal iron oxide NPs (250, 400,500, 750, and 1000µg/ml) with bacterial growth stock culture and sterilized brain heart infusion broth were prepared in test tubes. From this mixture, 200 µl from each dilution was spread on a 1 cm2 glass slide and incubated for 4 hours at 37 ºC. The slides were washed three times with D.W. In order to fix the adhered cells, 200 µl of methanol was flooded over each slide for 15 min. Thereafter, the non-adherent cells were removed by DW and 200 µl of crystal violet (1% w/v) was added to the slides and left for 15 min. The stained slides were washed with tap water, while the bounded stain was solubilized by 200 µl of (33% w/v) glacial acetic acid. The optical density of the solubilized stain was measured by an automated plate using an ELIZA reader at 630nm. Control preparation for each bacterial isolate was made as previously described under the same conditions and additions with the exception that bacterial suspension was replaced with distilled water (Zeraik and Nitschke, 2012).

Anti-Hemolysis assay

The effect of iron oxide (Fe3O4) NPs against bacterial hemolysis activity onto RBC was conducted using the same method described in (2.1.2) with little modifications as follows: A serial dilutions of previously prepared colloidal iron oxide NPs (500, 750, 900, 1000µg/ml) with bacterial growth stock culture, 200µl of previously prepared 5% RBC and brain heart infusion broth was prepared in test tubes. After 3 hours of incubation at 37oC, the mixture was centrifuged at 1500xg and the amount of hemoglobulin released in the supernatant was measured by spectrophotometer at 540 nm. The effect of iron oxide NPs against 5% RBC was also determined by using the same previously prepared serial dilutions but without the addition of bacterial growth suspension, instead D.W. and brain heart infusion broth were added to complete the volume.

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-27 (Statistical Packages for Social Sciences- version 27). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of the difference of

different means (quantitative data) was tested using Students-t-test for the difference between two independent means. The least significant difference (LSD) was used totest the significance of differences between different bacterial species. Statistical significance was considered whenever the P value was equal to or less than 0.05 (Daniel and Cross, 2018, Daniel, 2010).

RESULTS

Isolate bacteria and

Characterization and chemical synthesis of Iron oxide (Fe_3O_4) Nanoparticles were results by (Amin *et al.*, 2021).

Detection of bacterial virulence factors

Table 1: Detection of virulence factor (Biofilm, Hemolysin, and Adhesion) isolated bacteria

Bacteria isolated	Biofilm	Hemolysis	Adhesion
Staphylococcus aureus	1.922±0.127	0.776±0.054	0.291±0.064
Proteus mirabilis	1.592±0.181	0.516±0.121	0.259±0.067
Proteus mirabilis –standard	2.587±0.648	0.376±0.036	0.366±0.068
Enterococcus faecalis	1.760±0.246	0.760±0.073	0.346±0.055
Micrococcus luteus	0.956±0.057	0.956±0.057	0.288±0.064
Pseudomonas aeruginosa	1.599±0.169	0.119±0.024	0.460±0.058
Escherichia coli	1.576±0.236	0.985±0.095	0.144±0.067
P value	0.001^	0.0001^	0.001^
#The mean difference is significant at the 0.05 level using LSD for the difference between two independent means			
^The mean difference is significant at the 0.05 level using the ANOVA test for difference among more than two independent means			

Table 2: Different between gram-positive and gram-negative for each virulence factor (biofilm, hemolysis, adhesion)

Bacterial isolates	Biofilm	Hemolysis	Adhesion
Gram +ve	1.546±0.469	0.831±0.109	0.308±0.060
Gram –ve	1.839±0.549	0.499±0.336	0.307±0.135
P value	0.215	0.011	0.986
#The mean difference is significant at the 0.05 level using LSD for the difference between two independent means			

Results shown in Table (1), indicated that all three virulence factors (biofilm, hemolysis, and adhesion) were detected in 7 bacterial isolates Gram positive (*Staph aureus, Micrococcus luteus and Enterococcus faecalis)* and gram-negative bacteria (*E. coli, Proteus mirabilis standard, proteus mirabilis and P. aeruginosa)*. All bacterial isolates were able to produce (biofilm, hemolysis, and adhesion) but in different ratios with (P = o.oo1*), the highest biofilm formation levels were recorded in *Proteus mirabilis-2, Staph. aureus* (2.587±0.648, 1.922±0.127) respectively, while the highest (hemolysis) producing isolates were *E. coil* and *Micrococcus luteus* (0.985±0.095, 0.956±0.057) respectively. The high-level bacteria adhesion was determined in (*Pseudomonas aeruginosa* and *Enterococcus faecalis*) (0.460±0.058, 0.346±0.055) respectively. By making a comparison between gram-negative and gram-positive bacteria for producing those same virulence factors, results shown in table (2) estimated that gram-negative bacteria form biofilm (1.839±0.549) more than gram-positive with P value=0.215. While gram-positive bacteria hemolysis and adhesion activity (0.831±0.109, 0.308±0.060) were more than gram-negative bacteria with P values = (0.011 and 0.986) respectively.

The effect of (Fe3O4) NPs against bacterial virulence factors

Anti-biofilm formation assay

Table 3: The effects of Iron oxide nanoparticles on biofilm formation of *Staph aureus, Proteus mirabilis, and Proteus mirabilis standard)* at different concentrations

Concentrations of iron oxide nanoparticles with biofilm (µg/ml)	*Staphylococcus aureus*		*Proteus mirabilis*		*Proteus mirablis-standard*	
	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value
Control	0.333±0.005	-	0.451±0.016	-	0.466±0.013	-
250	0.318±0.008	0.681	0.456±0.204	0.952	0.582±0.018	*10.000
500	0.445±0.019	*0.007	1.338±0.074	*0.0001	0.844±0.019	0.0001*
750	0.324±0.097	0.805	0.995±0.145	*0.0001	0.508±0.034	0.022*
1000	0.332±0.058	0.978	0.426±0.006	0.762	0.469±0.027	0.855

5000	0.236±0.003	*0.017	0.218±0.029	*0.012	0.134±0.003	0.0001*
10000	0.134±0.016	*0.0001	0.200±0.008	*0.008	0.115±0.004	0.0001*
*Significant difference between control and different concentrations using Students-t-test at 0.05 level.						

The anti-biofilm formation ability of Iron oxide (Fe_3O_4) NPs against gram-positive *Staph. aureus*, *Proteus mirabilis*, and *proteus mirabilis-standard* gram-negative bacteria were determined because these isolated bacteria showed the highest biofilm formation ability as in table (3). Results shown in Table (3), indicated a variation in the amount of biofilm biomass formation during the 24h treatment time compared with the control. On *Staph aurous* the effect of nanoparticles is not statically significant (P=0.681) at the concentration 250 µg/ml (0.318±0.008), (P=0.805) at 750 µg/ml (0.324±0.097) and at 1000µg/ml (P=0.978) (332±0.058) but significantly at 500 µg/ml (0.445±0.019) (P=0.007*), (P=0.017*) at a concentration (5000 µg/ml (0.236±0.003), and (P=0.0001*) at 10000 µg/mg (0.134±0.016) as compared with the control. The effect of different concentrations of iron oxide nanoparticles on the biofilm formation ability of *Proteus mirabilis*, the effect was not statically significant (P= 0.952) at a concentration of 250 µg/ml (0.456±0.204), While significantly (P=0.0001*) at 500 µg/ml (1.338±0.074) and (P=0.0001*) at concentration 750 µg/ml (0.995±0.145), there was an increase in the ability of this bacteria at these concentrations to form biofilm as compared with control. In concentration 1000 µg/ml (0.426±0.006) not significantly (P=0.762), but significantly (P=0.012) 5000 µg/ml (0.218±0.029) and 10000 µg/ml (0.200±0.008) (P =0.008). There was a decrease in the biofilm at these concentrations as compared with control. For *Proteus mirabilis* -standard biofilm formation was affected statically significant at concentrations 250 µg/ml (0.582±0.018) (P=0.0001*), 500 µg/ml (0.844±0.019) (P=0.0001*),750 µg/ml (0.508±0.034) (P=0.022*), 5000µg/ml (0.134±0.003) (P=0.0001*),10000 µg/ml (0.115±0.004) (P=0.0001*), but not significantly (P=0.855) in 1000 µg/ml (0.469±0.027). In concentration (250,500,750,1000) µg/ml there was an increase in the level of biofilm compared with control, while in concentration (5000,10000) µg/ml there was a decrease in biofilm compared to control.

Anti-adhesion

The anti-adhesion ability of Iron oxide (Fe3 O4) NPs against gram-positive *Enterococcus faecalis*, and gram-negative *Pseudomonas aeruginosa*was determined because these isolated bacteria showed the highest adhesion ability in Table (3-1). In Table (3-4), there was a statically significant effect of NPs (P=0.511) on *Enterococcus faecalis* adhesion ability at a concentration of 500 µg/ml, but the higher level of adhesion compared to the control. Other concentrations (400 µg/ml, 750 µg/ml, and 1000 µg/ml) are not statically significant (P=0.511) (P=0.281) (P=0.547) respectively, an increase in adhesion at concentrations 400 µg/ml, 500 µg/ml and a decrease in adhesion at (750µg/ml and 1000µg/ml), as compared to control. In concentration 250 µg/ml the result is equal to control.

Table 4: The effect of different concentrations of iron oxide on adhesin for Enterococcus *faecalis* and *Pseudomonas aeruginosa* bacteria

Concentrations of iron oxide nanoparticles on adhesion (µg/ml)	***Enterococcus faecalis***	**P value**	***Pseudomonas aeruginosa***	***P value***
Control	1.160±0.148	-	1.120±0.211	-
250	1.160±0.148	-	1.190±0.123	0.600
400	1.280±0.151	0.511	1.180±0.159	0.653
500	1.550±0.381	0.048#	1.260±0.168	0.303
750	1.360±0.204	0.281	1.230±0.079	0.414
1000	1.270±0.115	0.547	1.130±0.182	0.940
*Significant difference between two independent means using Students-t-test at 0.05 level				
^Significant difference among more than two independent means using the ANOVA test at 0.05 level.				

In more detail, in Table (3-4), the anti-adhesion ability of iron oxide nanoparticles was not statically significant (P= 0.303) on *Pseudomonas aeruginosa* at a concentration of 500 µg/ml, but a higher level of adhesion compared to the control recorded. Other concentrations (400µg/ml,750 µg/ml, and 1000 µg/ml,) were not significantly statically (P=0.653) (P=0.414) (P=0.940) respectively, there was an increase in adhesion at concentrations (400 µg/ml and 500 µg/ml) and decrease adhesion in (750 µg/ml and 1000 µg/ml), as compared to the control. In concentration 250µg/ml there was an increase in adhesion as compared to control.

Anti-hemolysis

The effect of different concentrations of iron oxide nanoparticles on the hemolysis ability of bacterial isolates on RBC, two bacteria were chosen due to their highest hemolytic ability, *Micrococcus luteus* and *E. coli* as determined in table (1). In this experiment, only four concentrations 500µg/ml, 750µg/ml, 900µg/ml, and 1000µg/ml were prepared and used. The result

shown in Table (5), the anti-hemolytic ability of iron oxide nanoparticles was not statically significant (P=0.0001#) in all concentrations of iron oxide nanoparticles on bacterial hemolysis ability on human RBC as compared with control, but at concentration 1000 µg/ml of NPs the hemolysis ability of *Micrococcus luteus and E. coli* affected as compared with other concentrations. While, in *E. coli* the same result was shown at concentrations (of 500µg/ml, 750µg/ml, 900µg/ml and 1000µg/ml). Also, the highest hemolysis inhibition level was determined at 1000 µg/ml and the less inhibition at 500µg/ml as compared with the control (0.566±0.063).

Table 5: The effect of different concentrations of iron oxide nanoparticles on hemolysis abilities of *Micrococcus luteus and E. coli bacteria*

Concentration of iron-oxide nanoparticles on hemolysis (µg/ml)	*Micrococcus luteus*	P value	*E. coli*`	P value
Control	0.670±0.027	-	0.566±0.063	-
500	0.307±0.028	0.0001#	0.241±0.059	0.0001#
750	0.224±0.029	0.0001#	0.071±0.006	0.0001#
900	0.180±0.052	0.0001#	0.033±0.016	0.0001#
1000	0.118±0.017	0.0001#	0.010±0.003	0.0001#
**Significant difference between two independent means using Students-t-test at 0.05 level*				
^Significant difference among more than two independent means using ANOVA test at 0.05 level				

DISCUSSION

Detection of bacterial virulence factors

All the bacterial isolates were able to express their virulence factors, for (biofilm) in Table (3-1), and the highest biofilm formation levels were recorded in *Proteus mirabilis-standard* and *Staph. aureus* this is due to some virulence factors that have been linked to the ability of *P. mirabilis* to form crystalline biofilms. Such factors include swarming motility, fimbriae, urease production, capsule polysaccharide, and efflux pumps (Wasfi *et al.,* 2020). The encrustations on catheters are usually composed of struvite and apatite. Struvite (magnesium ammonium phosphate) forms large, often coffin-shaped crystals, and apatite (a hydroxylated form of calcium phosphate appears as microcrystalline aggregations. Scanning electron microscopy has revealed that large numbers of bacilli are associated with crystalline formations. Mixed populations of bacteria are commonly present and invariably contain species capable of producing the urease. Urease is the driving force of the crystallization process. It hydrolyses urea in the residual bladder urine to produce two molecules of ammonia to every molecule of carbon dioxide causing a rise in pH (Stickle *et al* ., 2014;Wasfi *et al.,* 2020). For virulence factor (Hemolysin), hemolysin production by Gram-positive and Gram-negative bacteria isolated from urinary tract infection was studied, and it was found that *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis, and Micrococcus luteus* have able to produce hemolysin as show a zone of β- hemolysis around colonies on a blood agar plate (AL-Hamawandi *et al*, 2014).

In table (3-1) secretion of the *E. coli* hemolysin (HlyA) has several distinctive features. These include a novel targeting signal located within the last 50 or so C-terminal amino acids, the absence of any periplasmic intermediates in transfer, and a specific membrane-bound translocator, HlyB, with important mammalian homologues such as P-glycoprotein (Mdr) and the cystic fibrosis protein. the nature of the HlyA targeting signal, the structure and function of HlyB, and the probability that HlyA is secreted directly to the medium through a trans-envelope complex composed of HlyB and HlyD (Holland *et al.,* 1990). Recently, a synergy between the insertion of HlyA in erythrocyte membranes and P2X receptors, ATP-activated ligand-gated cation channels that can increase plasma membrane permeability, was described as important for the action of HlyA to lyse erythrocyte membranes. Another indirect relationship occurs between K(Ca) channels and hemolysin. Upon permeabilization of the erythrocyte membrane by any pore-forming toxin, the flux in calcium activates K(Ca) channels and K^+ is exported from the cell. This response appears to benefit the host by causing erythrocyte shrinkage and recognition of the affected cell by phagocytic cells in the bloodstream to initiate clearance before lysis of the red blood cell occurs intravascularly. These results indicate that the channel formed by the toxin monomer or multimer is not the only site in the cell where ion fluctuations occur (Ristow *et al.,* 2016). Hemolysin has been described as a membrane toxin that damages the membrane of eukaryotic cells. In gram positive only one isolate of the genus *Micrococcus luteus* was beta-hemolytic and alpha-hemolysis and other species *S. aureus and E. faecalis* the alpha type toxin causes red blood lysis and destroys platelets, while the other type represents the beta type, which breaks down sphingomyelin and some other cells (Ghafoor and Yassin *et al.,*2020).

The infections caused by *Micrococcus subgroup* 3 were symptomatically severe, as were those caused by *Escherichia coli* (Kerr *et al.,* 1973). Virulence factor (adhesion) When pathogenic bacteria come into contact with intact human surface tissues (e.g., mucosa), they contrive to adhere to receptors on the surface of the target cells by means of various

surface structures of their own (attachment pili, attachment fimbriae, adhesion proteins in the outer membrane of Gram negative bacteria, cell wall-associated proteins in Gram-positive bacteria).This is a specific process, meaning that the adhesion structure (or ligand) and the receptor must fit together like a key in a keyhole. In the table (1), PavA like fibronectin-binding protein (EfbA) and adhesion to collagen of *E. faecalis* (Karimi *et al.,* 2018). *E. faecalis* is unable to bind to catheter material *in vitro* and is unable to grow in urine. This apparent paradox was resolved by the finding that urinary catheterization induces fibrinogen release into the bladder as part of the inflammatory response; this fibrinogen subsequently accumulates in the bladder and is deposited on the implanted catheter. Following fibrinogen deposition, the Ebp pilus adhesin — EbpA, which contains an N-terminal fibrinogen-binding domain — mediates catheter colonization and biofilm formation during CAUTIs caused *by E. faecalis.* Furthermore, *E. faecalis* can use fibrinogen for growth, enhancing biofilm formation on the catheter. This resolution of the paradox has been recapitulated in vitro by the demonstration that *E. faecalis* attaches to fibrinogen-coated catheters and grows in urine supplemented with fibrinogen (Flores-Mireles *et al.,* 2015) the first characterization of a PavA-like fibronectin-binding protein in *E. faecalis*, encoded by the EF1249 locus, which was termed enterococcal fibronectin-binding protein A (EfbA). We found that ΔefbA, an isogenic deletion mutant of the E. faecalis strain JH2-2, was abrogated in EfbA production thus resulting in a strongly diminished ability of the strain to bind to immobilized human fibronectin, providing strong evidence that EfbA plays a role in the pathogenesis of enterococcal UTIs (Torelli *et al.,* 2012).

When colonizing surfaces, *P. aeruginosa* relies predominantly on dynamic protein filaments called type IV pili that facilitate initial adhesion as well as migration and motility of the adhering bacteria. Each of these filaments is anchored in the inner membrane and consists of several thousand copies of a single protein subunit called pilin. These type IV pilins are structurally highly conserved in different *P. aeruginosa* strains and also other bacteria. Of particular medical importance is the C-terminal D-region, which contains a loop structure defined by a disulfide bridge and facilitates adherence to host-cell epithelial. In this study used to later Fe3o4 nanoparticles as antibacterial. So, choose the strongly biofilm strain from gram positive (*Staphylococcus aureus*) agreement (Shrestha *et al.,* 2019). Strongly strain from gram negative (*Proteus mirabilis slandered, proteus mirabilis),* high level hemolysis (*Micrococcus luteus*) from gram positive bacteria, high-level gram-negative bacteria *(E. coli*); and the high level to adhesion bacteria (*Pseudomonas aerogenes*) from gram negative and (*Enterococcus faecalis)* from gram positive bacteria. To express effect different concentration Fe3o4 to all virulence factor

The effect of (Fe3O4) NPs against bacterial virulence factors

Anti-biofilm formation assay

In table (1) iron oxide nanoparticles with engineered surface architecture and conjugated targeting ligands/proteins have attracted a great deal of attention for drug delivery applications. The fact that iron oxide NPs consist of cores made of iron oxides means they can be targeted to the required area through applying an external magnetic field the development of customized surfaces with specific properties, able to inhibit microbial attachment and biofilm formation in order to limit microbial colonization of medical surfaces and indwelling devices and to reduce mortality and morbidity associated with such severe infections (Holban *et al.,* 2016), biofilm can be considered a three-dimensional filter capable of capturing organic molecules, ions, and NPs. The interaction between NPs and biofilm can be considered as a three-stage process: (1) transfer of NPs in the vicinity of the biofilm; (2) attachment to the biofilm surface; and (3) migration in biofilms. Fe_3o_4 NPs against biofilms showed mostly insignificant effects. To obtain significant antibiofilm effects, Fe3O4 NPs have to be used in high level concentrations (1000,5000,10000 µg/ml). The particles are increase able to destroy the cells inside the biofilm. It has been shown that iron-oxide NPs were able to reduce biofilm growth by *S. aureus, E. coli, P. aeruginosa, S. epidermidis, and Enterococcus hirae*. In addition to the passive electrostatic effect on biofilm, NPs effectively penetrate deep into the biofilms in the presence of a magnetic field. In this case, NPs can have a mechanical effect on the biofilm due to the destruction of the matrix structure and its whole architecture (Shkodenko *et al.,* 2020). Increased IONPs concentration decreased biofilm formation. This results was compatible with (Abdul *et al*., 2019). In the Fe_2O_3 iron oxide NPs, 2nm particles did not inhibit biofilm growth but actually induced its formation. It was hypothesized. That these very small Fe_2O_3 NPs may have been an iron source for biofilm bacteria growth in this study in concertation (500 µg/ml) and (750 µg/ml) results demonstrate that iron oxide small particles increased growth and biofilm formation is linked to particle size, likely explained by the increased propensity of these smaller iron particles to readily dissolve more and release Fe^{3+} (aq) in to the aqueous medium. The current study is then in agreement with recent results showing that SPIONS (super paramagnetic iron oxide (Fe3O4) nanoparticles) also increase biofilm formation (Borcherding*et al.,* 2014; Abdul *et al*., 2019).

Anti-adhesion assay

For the result in table (4) Smaller NPs have larger specific surface areas, which result in higher probability of being in touch with and passing through the bacterial cell membrane than with larger NPs or polymers. Recent studies have demonstrated that the zeta potential of NPs has a strong influence on bacterial

adhesion. Because of the electrostatic attraction between positively charged NPs and the bacterial cell membrane, which is negatively charged, Mg (OH)2_MgCl and Mg $(OH)_2$_MgSO4 NPs, which have a positive surface charge, are prone to being adsorbed on the bacterial surface and are closely connected with bacteria, in contrast to their negatively charged counterparts. The potential of NPs to selectively gather at sites of bacterial infection increases vascular permeability. (Wang *et al.,* 2017) Agreement with (Asadishad B *et al*, 2013., Chifiriuc *et al* .2011). The adhesion of bacterial cells to naked as well as surface-modified ions was mainly through electrostatic and hydrophobic interactions. Since the contact between nanoparticles and the cell membrane is strong upon nanoparticle adhesion, bacteria may exhibit various metabolic responses. For example, (Borcherding*et al.,* 2014) reported increased bacterial growth (*Pseudomonas aeruginosa)* upon the attachment of IONs (Fe2O3) in a size-dependent manner where highest amount of growth was observed with the attachment of smaller IONs (2 ± 1nm) (Ranmadugala *et al*, 2018). The IO-NPs are rather exhibiting a stimulatory effect on the microbial growth especially at high concentrations. The stimulatory effect of iron oxide nanoparticles on the microbial growth was also reported by other authors, that is *E. coli, P. aeruginosa and E. faecalis, C. albicans*. These results could be explained by the ability of the microbial strains to use the iron oxide as a metabolic source of iron, which is known to positively regulcate the microbial growth rate and other physiological processes. (Prodan *et al*,. 2013).

Anti-Hemolysis assay

About the result in table (5), during the infection process, pathogens can secrete a variety of different toxins. These toxins may all participate in pathogenesis, but it is oftentimes hard to identify the most important. By incubating cell membrane-coated nanoparticles with bacterial protein secretions, multiple toxins with affinity to the source cell can be retained to form multi-antigenic nano toxoids. Hemolytic secreted proteins were first collected from the bacterial culture supernatant through ammonium sulfate precipitation, and the proteins were then incubated with the membrane-coated nanoparticles to fabricate the toxoids. Three toxins, including α-toxin, Panton Valentine leucocidin (PVL), and γ-toxin were confirmed to be present on the final formulation, and none of them were released significantly after washing or 48 h of dialysis. After the right-side out coating of cell membrane onto nanoparticulate cores, membrane components, including proteins, lipids, and glycans can be faithfully transferred to bestow desirable properties such as long circulation, tumor-homing, and many others. Usually, pathogen virulence factors such as pore-forming toxins must directly interact with target cells via their outer membrane. Leveraging this fact, cell membrane based nano sponges are able to serve as a generalized platform for toxin detainment. (Guo Zhongyuan *et al.,* 2021, Prodan *et al*, 2013). Hemolysin behaves like enzymes because it is a protein on which the effect of iron oxide. In this study, we focused on the key role of the ROS mediated mechanism in the implementation of their toxic effect, and studied the dose dependent effect of MNPs modified with various shells (based on polylactide, polysaccharide, or albumin) on ROS production by stimulated human blood cells and on the dynamics of induced oxidative hemolysis of erythrocytes. The immediate effect of these nanoparticle types on ROS production by blood cells in the absence of ROS inducing stimuli. For instance, we demonstrated that MNPs with a polysaccharide shell provided a dose dependent increase in ROS generation, while MNPs with a polylactide shell did not affect ROS production (Toropova YG *et al.,* 2021). And this fact disagrees with study which (LilitGabrielyan *et al.,* 2019).

CONCLUSION

Iron oxide nanoparticulate showed the ability to control some virulence factors against some Gram-positive and Gram-negative bacteria (*Staphylococcus aureus ،Enterococcus faecalis, Micrococcus luteus, proteus mirabilis، pseudomonas aeruginosa and Escherichia coli.*

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