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Effect of Ag_2O and TiO_2 Nanoparticles on *rsbA* Gene Expression Related to Swarming Phenomena in *Proteusspp.*

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Abstract: The *rsbA* gene is a regulatory gene associated with the swarming motility in *Proteusspecies*, which is linked to pathogenicity and survival mechanisms during urinary tract infections.The study aims to investigate the impact of silver oxide (Ag_2O) and titanium dioxide (TiO_2) nanoparticles on the *rsbA* gene expression associated with swarming phenomena in *Proteus spp*. A total of 109 urine specimens were collected from patients infected with urinary tract infections attending different hospitals in Baghdad, Iraq. Twenty-nine isolates were identified as *Proteusmirabilis*, while eleven bacterial isolates were identified as *Proteusvulgaris*. The Vitek-2 compact system was employed in order to confirm the primary bacterial identification. The bacterial ability for swarming movement was assessed using the central spot inoculation method for all *Proteusspp*. isolates. The minimal inhibitory concentration was determined using the agar dilution method; the results indicated that both *P. vulgaris* and *P. mirabilis* isolates were inhibited by Ag_2O nanoparticles at a MIC value of 10 mg/ml; on the other hand, TiO_2 nanoparticles inhibited the growth at 15 mg/ml. The disk diffusion assay was used to test antibiotic susceptibility. The results showed that about 60% of *P. vulgaris* and *P. mirabilis* isolates were not sensitive to ceftriaxone. Meropenem, on the other hand, was the most effective drug against *P. vulgaris* and *P. mirabilis*, with an 80% sensitivity rate. The ability of ten *Proteus* isolates for swarming movement in the presence of sub-inhibitory concentrations of nanoparticles (10 mg/ml and 5 mg/ml for TiO_2 and Ag_2O, respectively) was evaluated, and the results revealed that the swarming movement of all these isolates was decreased. *rsbA* gene expression was measured for ten *Proteusspp*. isolates before and after the addition of sub-inhibitory concentrations of nanoparticles using the qRT-PCR technique, and the results showed a decrease in the fold change values in most of the selected isolates, which refers to the downregulation effect on the *rsbA* gene related to swarming phenomena.The present study concluded that both Ag_2O and TiO_2 nanoparticles exhibit strong antibacterial activity against *Proteusvulgaris* and *Proteusmirabilis*, significantly reducing their swarming diameter.

Keywords: swarming phenomenon,*rsbA* geneexpression, *P. mirabilis, P. vulgaris*, , TiO_2NPs, AgNPs

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Introduction

The genus *Proteus* falls under the family *Enterobacteriaceae* and includes five recognized species: *P. mirabilis, P. vulgaris, P. penneri, P. myxofaciens*, and P. hauseri, in addition to three unnamed genotypes (1). Unlike their categorization as saprophytes in natural settings such as human microflora and the gastrointestinal tracts of animals, *Proteus spp*. are acknowledged as opportunistic pathogens linked to various infections. Their role as the third most common contributors to urinary tract infections has been

established(2). *P. mirabilis* and *P. vulgaris* are frequently implicated in urinary tract infections within complicated cases, particularly among individuals with indwelling catheters or structural abnormalities of the urinary tract (3,4). The multidrug-resistance observed in *Proteus spp*. necessitates ongoing evaluation of antimicrobial sensitivity patterns among clinically isolated strains. This is essential for making informed decisions regarding the appropriate antimicrobial agents to prescribe (5). On agar, the colonies of *Proteus. mirabilis* and*Proteus. vulgaris* exhibit an outward growth in a bull's-eye pattern, characterized by successive waves of rapid swarming that are subsequently followed by a consolidation into shorter cells (6). The phenomenon of swarming is frequently linked to the process of pathogenesis. Swarming bacteria demonstrated increased resistance to antibiotics and eukaryotic engulfment, while also acquiring improved nutrition and a competitive edge through the secretion of surfactants (7). Regulator of swarming behavior A (*RsbA)*serves as a regulatory gene for swarming phenomena, playing a crucial role in the survival of *Proteus*. It governs two distinct phases of the *Proteus* life cycle during urinary tract infections. Additionally, rsbA may act as a sensor for host defense response signals, regulating whether the colonizing swarmer cell exists in an adhesive or motile mode (8). The growing resistance of pathogens to antibiotics has led to significant health challenges in recent years. Nanoparticles are increasingly recognized as a promising alternative to antibiotics, showing significant potential in addressing the challenge of bacterial multidrug resistance. Specifically, silver nanoparticles (AgNPs) and titanium nanoparticles (TiNPs) have garnered significant interest within the scientific community (10). Silver and Titanium have historically been utilized to combat various diseases; they were previously employed as antiseptics and antimicrobials effective against both Gram-positive and Gram-negative bacteria (11). In years, AgNPs and TiNPs have emerged as particularly appealing options for developing a novel class of antimicrobials, paving the way for innovative strategies to address a diverse array of bacterial pathogens. The strong antibacterial and broad-spectrum effects against various microorganisms, both morphologically and metabolically distinct, appear to be linked to a complex mechanism through which nanoparticles engage with microbes. Furthermore, the specific structure and various methods of interacting with bacterial surfaces may present a distinctive and underexplored antibacterial mechanism to utilize(12–14).The study aims to investigate the impact of silver oxide (Ag_2O) and titanium dioxide (TiO_2) nanoparticles on the rsbA gene expression associated with swarming phenomena in *Proteus spp*.

Isolationand**identification of *Proteus spp.***

Patients with urinary tract infections at various hospitals in Baghdad, Iraq, provided a total of 109 urine specimens. We identified twenty-nine isolates as *Proteus* mirabilis. The bacteria were cultivated directly on blood agar and MacConkey agar. We then incubated the plates aerobically overnight at 37 oC for 24 hours. We identified the isolates using morphological characteristics and biochemical tests, following Bergy's Manual of Systematics of Archaea and Bacteria (15), and confirmed them using the Vitek-2 compact system.

Antibiotic susceptibility test

Colonies of purteriae cultivated on nutrient agar medium were moved into brain heart infusion broth and incubated at 37ºC for 4 hours to reach a turbidity standard comparable to that of a MacFarland tube; 0.5 corresponds to approximately a culture density of 1,5 x 108 cells/ml. Next, we evenly distributed the bacterial culture on Mueller-Hinton agar medium and allowed it to incubate for 10 minutes. Subsequently, the antimicrobial discs were placed on the agar medium. Following this, the plates were turned upside down and kept at 37ºC for 18 h (16). The zones of inhibition surrounding the discs were measured in millimeters (mm), and the isolate was categorized as susceptible, intermediate, or resistant to a particular drug by comparing it with standard inhibition zone measurements (17). All antibiotic discs were applied, including: Ceftriaxone 5µg, Meropenem 10µg, Amikacin 30µg, Gentamicin 10µg, Nitrofuraniton 300µg, Ciprofloxacin 5µg, and Cephalothin 30µg.

MIC Determination of Nanoparticles

The experiment was carried out according to (18) as follows: Different concentrations (5, 10, 15, 20, 25, and 30 mg/ml) were prepared from the stock solutions and mixed with blood agar and poured into Petri dishes. After that, a volume of 2 µl of an overnight bacterial culture with turbidity equals to McFarland standard no. 0.5corresponds to approximately a culture density of 1,5 x 10^8 cells/ml, was spotted onto the agar surface and incubated at 37°C for 24 h. The lowest concentration of nanoparticles in which there is no visible growth of the microorganism occurs is considered to be the MIC of the given isolate.

Investigate the anti-swarming effect of Ag_2O and TiO_2 Phenotypic effect

Phenotypic effect

The overnight culture (0.01ml) was inoculated centrally onto the surface of the dried blood agar plates with the presence of sub inhibitory concentrations of Ag_2O and TiO_2.after that the plates were incubated over night at 37ºC (19). The swarming diameters for the incubated isolates were measured and compared with the control that represent the plates without nanoparticles treatment.

RNA extraction

All selected isolates underwent incubation for 24 hours with sub-inhibitory concentrations of Ag_2ONPs and TiO_2 NPs nanoparticles. RNA was extracted from all samples utilizing the reagents supplied by GeneAid-South Korea. The RNA concentration and purity of each sample were assessed utilizing a spectrophotometer (Thermo Scientific).

cDNA synthesis, Evaluation of RT-PCR

For all samples except those designated for RNA-Seq, 1 µg of total RNA underwent reverse transcription into cDNA utilizing a high-capacity cDNA reverse transcription kit. RT-PCR was conducted using the 2 Power SYBR Green master mix. The reaction mixtures were composed of 2 SYBR green master mix, with each forward and reverse primer at a concentration ranging from 100 to 300 µM, and 25 µg of cDNA template. The final volume was adjusted to 25 µl using nuclease-free water. Reactions were conducted on the open qPCR following this protocol: Incubate at 50°C for 2 minutes, then proceed to 95°C for 10 minutes. Follow this with 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. An analysis of the dissociation curve was conducted for each assay to confirm product specificity (95°C for 15 s, 60°C for 1 min, 95°C for 15 s). The sequences for all primers are rpoAF (5'

GCGTGTTATAGCCCAGTTGA3') and rpoAR (5' AGGCTGACGAACATCACGTA 3') designated as the forward and reverse sequences for the housekeeping gene; rsbAF (5 CTATACCTACCGCACCATGT 3) and rsbAR (5' GAAGTCCCATCCGTTGATAC 3') identified as the forward and reverse sequences for the target gene(20). The change (fold) was determined using the 2CT formula, withrpoA utilized as the housekeeping gene.

Results and discussion

Isolation and identification

One hundred and nine urine specimens from patients with urinary tract infection attending different hospitals at Baghdad city were collected through the period from September 2022 to January 2023. forty (36%) isolates were primarily identified as *Proteus spp*. basing on its biochemical properties that illustrated in in (Table1), as well as their ability for swarming movement with no hemolytic activity on blood agar media. 29 (72.5%) isolates were*P. mirabilis* and 11 (27.5%) isolates were *P. vulgaris*. The identification was then comferd by using VITEK 2 compact system.

Swarming motility assay

The ability of all *Proteus* isolates (40) for swarming movement was evaluated on blood agar plates and the diameters of swarming were measured in millimetres. The result revealed that there was a great variation in their swarming behaviour. There is a large variation in swarming diameter in all *P. vulgaris* isolates which extended from 6mm to 31mm on blood agar plates and also there is a variation in all *P. mirabilis*isolates that ranged from 8 to 44 mm in all plates. Ten isolates (5 isolates *P. vulgaris* and 5 isolates *P. mirabilis*) were selected with variable swarming diameter (3 weak, 3 intermediate and 4 strong) for sub-sequent experimentation similar studies byBudding et, al the result of study show *Proteus species*, including *P. mirabilis* and *P. vulgaris*, are known for their distinctive swarming motility on agar surfaces14. This swarming behavior is characterized by: The formation of concentric rings of growth on agar plates (21).

Antibiotic susceptibility test

The selected isolates were tested for their susceptibility toward (Amikacin, ceftriaxone, cephalothin, Ciprofloxacin, Gentamicin, Nitrofurantion, meropenem); the results presented in Figure 1 reveals a similar susceptibility pattern of *P. vulgaris* isolates toward Cephalothin and Gentamicin on one hand and in the other hand towards Ciprofloxacin and Meropenem; while it showed different susceptibility pattern towards other antibiotics. The highest resistance percentage was found to ceftriaxone (60%). Moreover, (40%) of the *P. vulgaris* isolates were resistant to Amikacin and Nitrofurantion. On the other hand, only (20%) of *P. vulgaris* isolates were resistant to cephalothin, Ciprofloxacin, Gentamicin and meropenem. Furthermore; (20%) of the isolates developed intermediate resistance towards Amikacin, ceftriaxone, cephalothin and Gentamicin. Nevertheless; Meropenem and Ciprofloxacin revealed be the most effective drugs since they recorded the highest sensitivity percentage of 80%. In regard to *P. mirabilis* isolates, the results summarized in Figure 2 reveals a similar susceptibility pattern of *P. mirabilis* isolates toward Amikacin, cephalothin, Ciprofloxacin and Nitrofurantion, with 20, 20 and 60% being resistant, intermediate and sensitive respectively. The highest

resistance percentage was also found to ceftriaxone (60%). Moreover, (40%) of the *P. mirabilis* isolates were resistant to Gentamicin. On the other hand, none of *P. mirabilis* isolates were resistant to meropenem, though (20%) of the isolates developed intermediate resistance making Meropenem as also the most effective drug.

The antibiotic susceptibility testing revealed that *Proteus spp*. exhibit a broad spectrum of resistance to various antibiotics. This could arise from the presence of an extra outer cytoplasmic membrane, consisting of a lipid bilayer, lipoproteins, polysaccharides, and lipopolysaccharides. Furthermore, the inappropriate use and overuse of antibiotics may contribute to the emergence of resistance to these drugs (22). The consistent use of antibiotics in human and animal healthcare has resulted in a notable rise in antibiotic resistance and the development of antibiotic resistance genes, especially within gram-negative organisms. (23,24).

Determination of minimal inhibitory concentration (MIC) for Ag_2O and TiO_2 nanoparticles

The susceptibility of the bacterial isolates (*P. vulgaris* and *P. mirabilis*) towards Ag_2O and TiO_2 nanoparticles was tested, and the MIC values were determined using agar dilution method. Different ascending concentrations of Ag_2O and TiO_2 nanoparticles (5, 10, 15, 20, 25 and 30 mg/ml) were mixed with Muller hinton agar and poured into Petri dishes for this purpose. From the findings of the present study, the results were revealed that both *P. vulgaris*and *P. mirabilis*isolates were inhibited by Ag_2O nanoparticles at fixed MIC value of 10 mg/ml; on the other hand, 15 mg/ml TiO_2 nanoparticles was considered MIC for the growth of *P. vulgaris* and *P. mirabilis* isolates, by treating the bacteria with these nanoparticles concentrations it shows no growth as shows in tables 1, 2, 3 and 4.

The result agrees with a local study by Al-Bahrani and Ghafil who stated that Ag_2O nanoparticles with a concentration of 25µg/ml demonstrated an inhibitory effect with inhibition zone diameters of 25 and 28 mm against *P. mirabilis* and *P. vulgaris* respectively using well diffusion method28. A study by Ibrahem and Salman investigated biologically synthesized titanium nanoparticles, revealing that these TiO_2 nanoparticles exhibited significantly higher antimicrobial efficacy against multidrug-resistant bacteria responsible for recurrent urinary tract infections, including *Klebsiellapneumoniae*, *Staphylococcus* aureus, *Acinetobacterbaumannii*, *E. coli*, and *Morganellamorganii*. The study also noted the impact on virulence factors such as biofilm formation, hemolysin, and urease after treatment with sub-MIC (16 mg/ml) titanium nanoparticles. Noomi et al. conducted a study on the bactericidal effects of silver (Ag) and titanium (Ti) nanoparticles against various species of multidrug-resistant bacteria isolated from sheep wound infections. They reported that both nanoparticles exhibited inhibitory effects, with inhibition zones measuring 18 mm for Ag and 20 mm for Ti nanoparticles, respectively (25). Ag+ ions have been shown to disrupt respiratory electron transport from oxidative phosphorylation, inhibit enzymes in the respiratory chain, or affect membrane permeability to protons and phosphate (26,27). Moreover, elevated concentrations of Ag+ ions have demonstrated interactions with cytoplasmic components and nucleic acids (28). The antimicrobial properties of TiO_2 are associated with its crystal structure,

morphology, and dimensions. Oxidative stress resulting from the generation of reactive oxygen species (ROS) is proposed as a significant mechanism associated with TiO_2 nanoparticles in their anatase forms. Reactive oxygen species induce site-specific DNA damage (29,30). The photocatalytic properties of TiO_2 nanoparticles enable efficient bacterial eradication. TiO_2 nanoparticles generate reactive oxygen species when exposed to UV light, which contributes to antibacterial photocatalytic activity. This process is associated with lipid peroxidation, leading to increased membrane fluidity and disruption of cell integrity (12,31)

Determining the anti-swarming effect of Ag_2O and TiO_2 nanoparticles

The ability of ten *Proteus* isolates for swarming movement in the presence of sub-inhibitory concentrations of nanoparticles (10 mg/ml and 5 mg/ml for TiO_2 and Ag_2O respectively) was evaluated as illustrated in (Figure 3&4).

The decrease in swarming movement in the presence of nanoparticles could be attributed to their potential role in impairment of flagella syntheses or rotation and thus their activity which is necessary for cellular movement of *Proteus spp*. or may be mutations causing defects in flagella synthesis or flagella function must abolish colony spreading the mutations that reduce flagellar gene expression reduce flagellar number and reduce or abolish swarming (32), There are many reasons behind the variation in swarming movement for *Proteus spp*. And these reasons may be exhibited with bacteria themselves including the slight differentiations between the strains of the same genus and / or the environmental conditions surrounded the growing bacteria or the expression of particular genes that related to swarming movement (33,34). As for as we know; There are presently no studies about the TiO_2NPs and Ag_2O NPs effect of on the swarming movement of *Proteus spp*, however, there are several articles regarding the effect of synthesized the TiO_2NPs and Ag_2O NPs as antimicrobial agent against *Proteus spp*(35,36).

Determining the effect of nanoparticles on *rsbA* gene expression

The effect of sub-inhibitory concentrations of the sub-inhibitory concentration of TiO_2andAg_2O nanoparticles on the expression levels of *rsbA* gene was assessed using Rt-PCR in the selected *P. vulgaris* and *P. mirabilis* isolates, the results indicates that there was a down regulation effect in the *rsbA* gene expression levels in must *P. mirabilis* and *P. vulgaris* isolates. The fold change values express the alternation in the expression manner of *rsbA* gene after 24 hr of incubation in the presence of TiO_2andAg_2O nanoparticles, the decreasing in the fold change values for the treated isolates express the down regulation effect of the nanoparticles phenotypically by reducing the swarming diameters and genetically by reducing the gene expression Table 5. In other word, there is a strong relationship between the *rsbA* gene and the swarmingphenomena and both of them are effected by nanoparticles and this effect may be related to the antimicrobial activity, another study by Wang, et.al, Membrane damage, Nanoparticles can disrupt bacterial cell membranes, leading to loss of integrity and cell death (37) chemical and physical properties of nanoparticles that may interfere with different extra and intra cellular proteins of the bacterial cells such as flagellar protein and bacterial membrane and cell wall caused blocking in their bacterial activities (38). Also it might be because a damage

in internal parts of bacteria, ions liberation, and oxidative stress and DNA damage the study of Mondal, et, al Interference with protein function: Nanoparticles can interact with various bacterial proteins, including those involved in flagellar movement and membrane function (39).

Table (1): The minimum inhibitory concentrationvalues for Ag_2O NPs nanoparticles vs *P. mirabilis*

Isolates	AgNPs nanoparticles concentrations mg/ml					
	5	10	15	20	25	30
P.m1	o	X	x	x	x	x
P.m2	o	X	x	x	x	x
P.m3	o	X	x	x	x	x
P.m4	o	X	x	x	x	x
P.m5	o	X	x	x	x	x
P.m6	o	X	x	x	x	x
P.m7	o	X	x	x	x	x
P.m8	o	X	x	x	x	x
P.m9	o	X	x	x	x	x
P.m10	o	X	x	x	x	x

* (x) inhibition of bacterial growth
* (o) means bacterial growth.

Table (2): The minimum inhibitory concentration values for TiO_2 nanoparticles vs *P. mirabilis*

Isolates	Tio2 nanoparticles concentrations mg/ml					
	5	**10**	**15**	**20**	**25**	**30**
P.m1	o	O	x	x	x	x
P.m2	o	O	x	x	x	x
P.m3	o	O	x	x	x	x
P.m4	o	O	x	x	x	x
P.m5	o	O	x	x	x	x
P.m6	o	O	x	x	x	x
P.m7	o	O	x	x	x	x
P.m8	o	O	x	x	x	x
P.m9	o	O	x	x	x	x
P.m10	o	O	x	x	x	x

* (x) inhibition of bacterial growth
* (o) means bacterial growth.

Table (3): The minimum inhibitory concentration values for Ag_2O NPs nanoparticles vs *P. vulgaris*

Isolates	AgNPs nanoparticles concentrations mg/ml					
	5	**10**	**15**	**20**	**25**	**30**
P.v 1	o	x	x	x	x	x
P.v 2	o	x	x	x	x	x
P.v 3	o	x	x	x	x	x
P.v 4	o	x	x	x	x	x
P.v 5	o	x	x	x	x	x
P.v 6	o	x	x	x	x	x
P.v 7	o	x	x	x	x	x
P.v 8	o	x	x	x	x	x
P.v 9	o	x	x	x	x	x
P.v 10	o	x	x	x	x	x

* (x) inhibition of bacterial growth
* (o) means bacterial growth.

Table (4): The minimum inhibitory concentration values for TiO_2 nanoparticles vs *P. vulgaris*

Isolates	Tio2NPs nanoparticles concentrations mg/ml					
	5	10	15	20	25	30
P.v 1	o	O	x	x	x	x
P.v 2	o	O	x	x	x	x
P.v 3	o	O	x	x	x	x
P.v 4	o	O	x	x	x	x
P.v 5	o	O	x	x	x	x
P.v 6	o	O	x	x	x	x
P.v 7	o	O	x	x	x	x
P.v 8	o	O	x	x	x	x
P.v 9	o	O	x	x	x	x
P.v 10	o	o	x	x	x	x

* (x) inhibition of bacterial growth
* (o) means bacterial growth.

Table (5): Fold change values for *P. mirabils* and *P. vulgaris*

The isolates bacteria (5 for each subtype)		Control		AgNO3		TiO_2	
		rpo	***rsba***	***rpo***	***rsba***	***rpo***	***rsba***
P. mirabilis	M1	14.68	16.54	16.04	0	21.36	29.39
	M2	17.33	12.75	21.62	30.08	13	29.14
	M3	22.85	28.45	13.68	27.4	15.36	28.3
	M4	14.18	17.89	19.68	26.49	13.97	28.76
	M5	31.27	11.84	13	28.47	19.35	28.33
P. Vulgaris	V1	17.13	32.43	14.28	27.6	13.95	29.35
	V2	12.43	15.05	13.6	27.55	14.48	31.93
	V3	12.08	14.27	13.58	28.37	11.25	28.11
	V4	14	27.63	12	28.84	14.1	25.57
	V5	14.52	17.6	14.14	28.63	14.3	26.29

rpo which is considered as a house keeping gene in this study

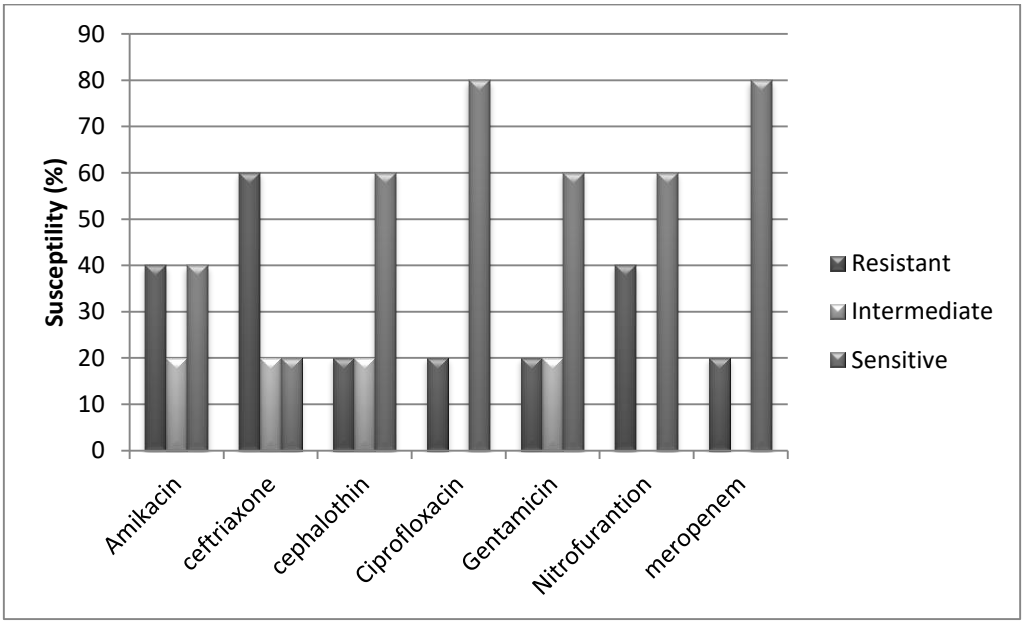

Figure (1): Antibiotic susceptibility of 5 isolates *P. vulgaris*.

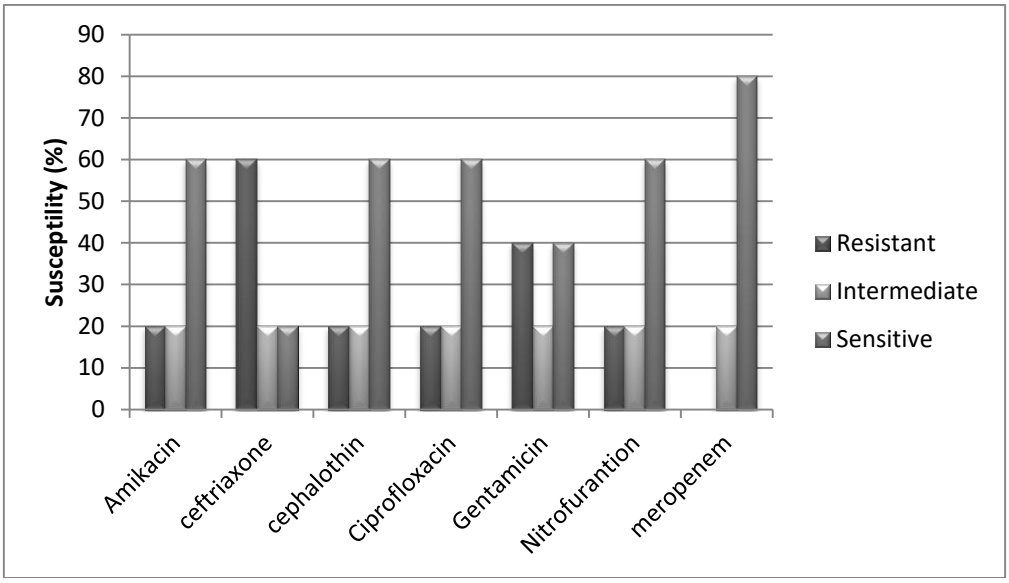

Figure (2) : Antibiotic susceptibility of 5 isolates *P. mirabilis*

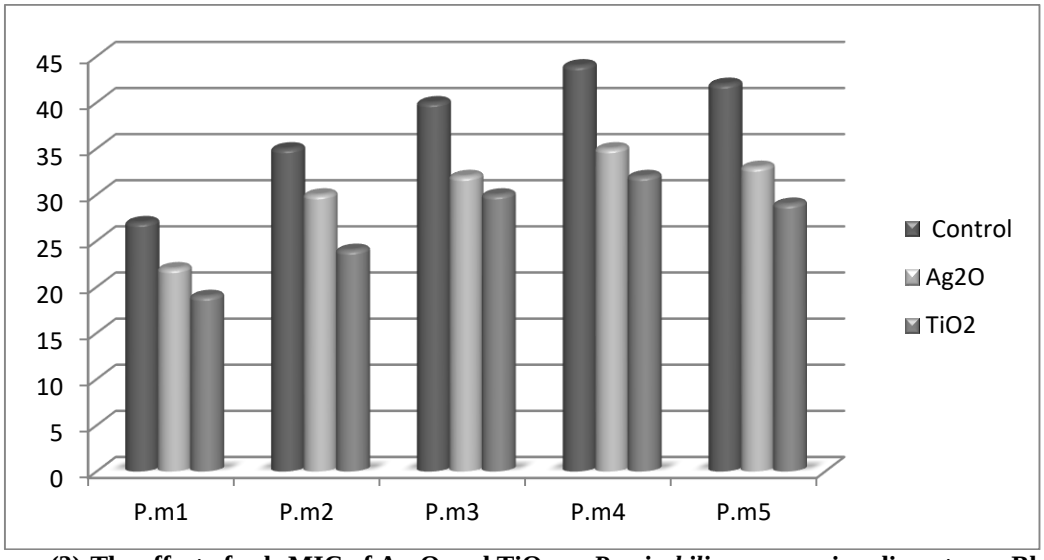

Figure (3):The effect of sub-MIC of Ag_2O and TiO_2 on *P. mirabilis*on swarming diameteron Blood agar

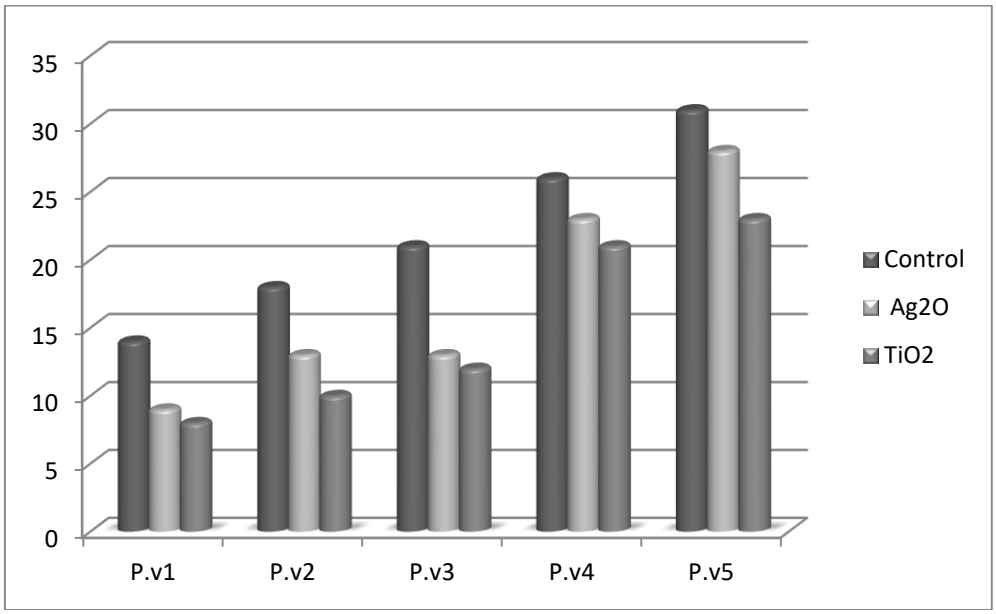

Figure (4) :The effect of sub-MIC of Ag_2O and TiO_2 on *P. vulgaris*on swarming diameteron Blood agar

Conclusion

The present work has reached these conclusions:The results demonstrated that both nanoparticles possess strong antibacterial properties, significantly reducing swarming diameter in *Proteus vulgaris* and *Proteus mirabilis*. Furthermore, the sub-inhibitory concentrations of these nanoparticles induced a downregulation of *rsbA* gene expression, suggesting a dual action in physically impeding bacterial movement and genetically reducing swarming capability. This dual mechanism enhances the potential of Ag_2O and TiO_2 nanoparticles as alternative therapeutic agents against multidrug-resistant strains of *Proteus*, offering a novel approach to combat persistent urinary tract infections.

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