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Research Article

Evolution of Antibiotic Resistance in Gram-Negative Uropathogens and Effect of Bio-Cobalt oxide Nanoparticles as Antibacterial and Antbiofilm Agents

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A B S T R A C T

Background: Urinary tract infections are among the most common infections worldwide. As the rise of antibiotic resistance in uropathogenic bacteria has made treatment more challenging, nanoparticles are being explored to treat these infections. This study aimed to synthesize and evaluate the antibacterial activity of cobalt oxide nanoparticles from *Hibiscus sabdariffa* flowers against multidrug-resistant Gram-negative uropathogens.

Materials and Methods: 100 urine samples were collected from patients with urinary tract infections at Tikrit hospital in Iraq. Bacteria were cultured on MaCconky, EMB agar and identified using Vitek-2 system. This was followed by a sensitivity test for seven antibiotics, and detection of biofilm formation. Cobalt oxide nanoparticles were synthesized, and analyzed using scanning electron microscopy (SEM), FTIR spectroscopy, and UV-Vis spectroscopy. Their efficacy against the bacterial isolates was then evaluated at different concentrations (25%, 50%, 75%, and 100%).

Results: Gram negative bacteria were detected in 57 isolates (57%). These included *E.coli* (34 isolates, 59.64%), *Klebsiella pneumoniae (*17 isolates, 29.82%), *Pseudomonas aeruginosa* (4 isolates 7.01%), and *Proteus mirabilis* (2 isolates, 3.5%). Ten isolates - five isolates of *E. coli*, two isolates of *P. aeruginosa*, and three isolates of *K. pneumoniae* - exhibited 100% resistance to antibiotics seven type used in this study also these isolates produce strong biofilm. The characterization of Bio-Cobalt oxide nanoparticles detect through used UV-Vis spectroscopy showed high absorbance at 512 nm, while scanning electron microscopy appered sizes of cobalt oxide nanoparticles ranging from 50.06 to 97.41 nm, a spherical shape, FTIR analysis showed peaks from 563.21 cm^{-1} to 3259.70 cm^{-1}. Assessment of the antibacterial activity of cobalt oxide nanoparticles showed inhibitory action at diameters of 27-32

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mm against five isolates of multi drug resistant *E. coli*, 23-24 mm against two isolates of *P. aeruginosa*, and 25-26 mm against three isolates of *K. pneumoniae.* Antibiofilm activity was also recorded.

Conclusion: Biological method of synthesis Cobalt oxide nanoparticles using plant H. sabdariffa extract was eco-friendly and safety method The Cobalt oxide nanoparticles (CoNPs) exhibited potent biological activity against multidrug-resistant (MDR) Gram-negative bacteria.

Keywords: Cobalt oxide Nanoparticles, Gram-negative bacteria, Urinary tract infections

Introduction

Urinary tract infections (UTIs) caused by bacteria are among the most common infections, affecting millions of people worldwide.[1] Globally, between 150 and 250 million cases are reported annually, indicating high infection rates and resulting in high treatment costs.[2] UTIs, which are a serious health problem, may be caused by a wide range of pathogens, including Gram-negative and Gram-positive bacteria and yeasts. These pathogens can infect any part of the urinary tract, including the bladder, ureters, urethra, or renal pelvis.[3] As antibiotic resistance has emerged as major challenge in treating UTIs caused by bacteria, there is a growing need to find new therapeutic alternatives. In this context, nanotechnology has emerged as a promising approach.[4]

In the last few years, the application of nanoscale technologies and nanoparticles has led to innovation of new methods to eliminate multidrug-resistant bacteria for this purpose, various nanoparticles with diameters ranging from 1 to 100 nanometers have been synthesized.[5]

Cobalt oxide nanoparticles have shown a strong potential in treating pathogenic bacterial, fungal, or amoebic infections.[6] Recent studies have indicated the effectiveness of cobalt oxide nanoparticles against bacteria.[7] Since these can be manufactured in several ways, cobalt oxide nanoparticles have attracted more economic interest than other noble metal nanoparticles. Researchers have focused on developing ecologically friendly, sustainable, and clean synthesis techniques.[8] for these. The use of plants to synthesize cobalt oxide nanoparticles is promising due to availability of various structural parts and their ability to store energy.[9] A variety of plant components, such as seedlings, leaves, peelings, stems, roots, fruits, and interior parts, can be used to create cobalt oxide nanoparticles.[10]

Nanoparticles synthesized by plants are easy to obtain, safe to handle, and have a variety of bioactive substances such as terpenoids, alkaloids, phenols, tannins, flavonoids, and quinines that aid in their creation.[11,12] Among the plants which are a source of nanoparticles is Hibiscus. It contains several active chemical compounds represented by organic and phenolic acids including citric, malic, succinic, and lactic acids, in addition to flavonoids, as phenolic compounds and flavonoids, which play a role in antioxidant and antimicrobial activity, and anthocyanin, which accounts for the bright color of flowers. The chemical composition of plant Hibiscus varies according to the type, origin, age, and color.[13] This study aimed to synthesize and characterize cobalt oxide nanoparticles using H. sabdariffa extract and investigate their effectiveness against multidrug-resistant bacterial isolates.

Materials and Methods

Study design

This study performed from February to August 2025 at Tikrit Teaching Hospital in Salah al-Din Governorate, Total One hundred urine samples were collected from patients suffering from urinary tract infections , The samples were obtained from both sex include 60 women and 40 men and across an age range from 5 to 70 years, This took place following the clinical diagnosis of symptoms and signs by doctor for each case, the urine sample collected from patients with some criteria include The patient must not be taking any antibiotics and must be free from infectious diseases, The samples were collected using the "midstream" method in sterile, tightly sealed containers to prevent leakage, All urine samples were transported to the lab within one hour and diagnosis according to microbiological and biochemical tests after cultured on MacConkey, Blood, Cetrimide Agar and EMB media(Hi media –India). Diagnosis was confirmed by using VITEK-2 technique (BioMérieux, France).

Antibiotic Susceptibility Test

The disk diffusion method, as described by[14, 15] was used to examine the susceptibility of bacterial isolates to seven types of antibiotics (Table 1). After the bacterial count was adjusted by comparison with the 0.5 McFarland standard, single colonies of each bacterial species were spread on to the surface of Mueller–Hinton agar (Oxoid, England) using a sterile swab. The inoculated plates were then allowed to stand for five minutes, after which antibiotic discs were placed on the plate surface using sterile forceps. Following a 24-hour incubation period at 37 °C, the diameter of the growth inhibition zone was measured using a ruler. The results were interpreted according to CLSI guidelines.

Table 1.Antibiotics discs used to detect bacterial resistance

Antibiotic Type	Symbol	Concen tration	Manufacture Company
Meropenem	MEM	30µg	Bioanalyse-Turkey
Nitrofurantoin	F	100 µg	Bioanalyse
Amikacin	AK	10 µg	Bioanalyse
Cefepime	FEP	30 µg	Bioanalyse
Gentamicin	CN	30µg	Bioanalyse

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Trimethoprim-sulfamethoxazole	SXT	25 µg	Bioanalyse
Ciprofloxacin	CIP	10 µg	Bioanalyse

Detection of Biofilm formation

Two method used to detect bacterial isolates to produce biofilm include

Congo red Agar Method (CRA) (Qualitative Method)

The culture medium was prepared according to[16] the components: heart-brain infusion (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo red day (0.8 g/L). The day was prepared as an aqueous solution and sterilized separately from the other components at 121°C for 15 minutes. Subsequently, the dye was added to the remaining sterilized components after they had cooled to 55°C, and the prepared medium was poured into sterile Petri dishes. Once solidified, the plates were inoculated with a suspension of the bacterial samples using a sterile loop and incubated at 37°C for 24 hours under aerobic conditions, after that results appeared through morphology of colony, Black colonies with crystalline consistency indicated production of biofilms while red colonies indicated a negative.

Microtiter Plate Assay (Quantitative Method)

Microtiter plate method which utilizes a sterile 96-well plate, was used to detect the ability of bacteria to produce biofilms, according to the method described by.[17]

The all bacterial isolates in this study were inoculated into test tubes(tube each bacterial isolate) containing liquid nutrient broth and incubated at 37°C for 24 hours. Then 200 µL of the bacterial suspension (1×10^8) was transferred into three wells in a microtiter plate (MTP) for each bacterial isolate; un inoculated nutrient broth served as a control. The plate was covered and incubated at 37°C for 24 hours under static conditions. Following incubation, the well contents (bacterial suspension) were removed via pipette, and the wells were washed three times with 200 µL of phosphate-buffered saline (PBS, pH 7.2). to remove non-adherent cells. After allowing the wells to air-dry, they were stained with 200 µL (1% w/v) crystal violet for 30 minutes, washed with sterile distilled water, and left to air-dry; the amount of dye adhering to the wells allowed for the assessment of bacterial attachment ability. To quantitatively estimate the bacteria's ability to produce biofilm, the bound dye was extracted by adding 200 µL of 95% ethanol, and absorbance was measured at 595 nm using a microplate reader. The experiment was performed in triplicate, and the following values were used to categorize the biofilm-producing potential of the isolates:

- < 0.120: Non-biofilm producer
- 0.120–0.240: Weak biofilm producer
- > 0.240: Strong biofilm producer

Preparation of Plant Extract

The petals of Hibiscus were obtained from local markets and washed with sterile distilled water. They were then dried and ground into a fine powder using a blender. The plant extract was prepared according to the process detailed by[18], with some modifications. Two grams of hibiscus petal powder were weighed and transferred to a 250 mL glass flask containing 100 mL of sterile distilled water. The mixture was heated for one hour at 60 °C and then left to cool at room temperature. It was subsequently filtered through Whatman No. 1 filter paper (pore size: 25 µm) and stored in the refrigerator until use.

Preparation of Cobalt oxide Nanoparticles

The procedure was carried out as described by[19, 20], with some modifications. A total of 15 mL of plant extract was slowly added to 35 mL of a 0.01 M $Co(NO_3)_2{\cdot}6H_2O$ (cobalt nitrate) solution, which was prepared by dissolving 2.91 g of cobalt nitrate in 100 mL of distilled water to achieve a 1 molar concentration. The solution was mixed thoroughly and stored in an opaque bottle to prevent oxidation of cobalt nitrate. The mixture was then stirred continuously on a hot plate at 60 °C for 15 minutes until the desired color change was observed, the Stock solution of Cobalt oxide nanoparticles 100% prepare as solution 100 ml (volume) Other concentrations of Cobalt oxide nanoparticles were prepared from the stock solution include

- Concentration (25) % Cobalt oxide nanoparticles contain 25 ml Cobalt oxide nanoparticles solution + 75 ml sterilize deionized water
- Concentration (50) % Cobalt oxide nanoparticles contain 50 ml Cobalt oxide nanoparticles solution + 50 ml sterilize deionized water
- Concentration (75) % Cobalt oxide nanoparticles contain 75 ml Cobalt oxide nanoparticles solution + 25 ml sterilize deionized water

Characterization of Cobalt oxide Nanoparticles

UV–Visible Spectroscopy

UV–Vis spectral analysis was used to confirm the formation of cobalt oxide nanoparticles. The device was calibrated prior to analyzing the sample by transferring 2 mL of the solvent used in the experiment—deionized water—into a clean cuvette, placing the cuvette in the device, after that analyzing the sample. two ml of cobalt nanoparticles solution shaken well to homogenize transfer by pipet to quartz cuvette Place the cuvette into the instrument's sample holder, ensuring it is correctly oriented relative to the light path; then, close the instrument lid and operate the device to perform a spectral scan a wavelength range of 300–800 nm., The analysis was performed using a UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan)[21]

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Scanning Electron Microscopy (SEM)

The morphology, distribution, and size of cobalt oxide nanoparticles were determined by scanning electron microscopy. The nanoparticle powder (100 mg) was dried at 45 °C in an oven (Gallenkamp, England) The powder was deposited onto a flat plate, and the particles were coated with a thin layer of gold to prevent charge buildup during the vacuum process. The sample was then transferred to the instrument, where an electron beam was directed at its surface; the resulting signals were collected to display a magnified, detailed image of the sample on the instrument's screen.[21, 22]

Fourier-Transform Infrared Spectroscopy (FTIR)

One milligram of the nanoparticle powder is added to 100 milligrams of dry potassium bromide (KBr) powder; the mixture is ground together using a ceramic mortar and pestle for three minutes to obtain a completely homogeneous, fine powder, and is then transferred to a steel mold to be pressed into a pellet., transfer pellet to Sample Holder of instrument FTIR spectrometer (Shimadzu, Japan). The device emits radiation directed at the sample, and the result appears as curves on the screen, the range between 600–4000 cm^{-1}.[21, 22]

Antibacterial Activity of Cobalt oxide Nanoparticles

The antibacterial effect of cobalt oxide nanoparticles against the multidrug-resistant bacterial isolates (ten isolates) was evaluated using well diffusion method, as described by.[23] Of the ten isolates that exhibited 100% resistance to seven antibiotics in the study, five E. *coli*, two were P*. aeruginosa*, and three were *K. pneumoniae*. The bacterial count was adjusted to 1×10^8 CFU/mL using McFarland standards. Using a sterilized cotton swab, the bacterial suspension was spread evenly over the surface of Mueller–Hinton agar. The plates were left to stand for 15 minutes, after which wells of uniform dimensions (6 mm diameter) were made using a sterile cork borer. Subsequently, 100 µL of each concentration of cobalt oxide nanoparticles (25%, 50%, 75%, and 100%) was transferred into the respective wells using a micropipette, and 100 µL of sterile distilled water was added to one well as a negative control. The Petri dishes were incubated for 24 hours at 37 °C. Following incubation, the diameters of the inhibition zones that had developed around the wells were measured in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[24]

Antibiofilm Activity of Cobalt oxide Nanoparticles

Congo red agar method

To assess the anti-biofilm activity, 120 µL of a fixed bacterial suspension (1×108) was added to 80 µL of Cobalt oxide nanoparticles in various concentrations (25%, 50%, and 75%) in sterile test tubes (one tube for each concentration). The tubes were incubated for 24 hours at 37°C. Five microliter from each tube was transferred on to Congo red plates using sterile loop which were then incubated for 24 hours at 37°C. [25]

Microtiter plate method

Cobalt oxide nanoparticles' activity against formation of bacterial biofilms was detected using the method described by [26] with modifications, A microliter -well plate was used to detect the efficacy of various concentrations of cobalt oxide nanoparticles in inhibiting biofilm formation by bacterial isolates, A 150 microliter of the bacterial suspension ten isolate of bacteria (concentration of 0.5 McFarland) was transferred into each well. Subsequently, 50 microliter of three different cobalt oxide nanoparticle concentrations (25%, 50%, and 75%) added to wells of microliter plate. Additionally, 200 µL of sterile distilled water add to one well as a negative control, while 200 µL of the bacterial suspension without nanoparticle additives (one well per isolate) used as a positive control to detect the bacteria to biofilm-forming capability. The plate was then incubated at 37°C for 16 hours. Following incubation, solution in each well were removed, then washed with phosphate-buffered saline (PBS) and allowed to air-dry. Subsequently, 200 µL of a 1 % crystal violet solution was added to each well and left for 5 minutes; the dye was then removed, and the wells were washed twice with buffer solution and allowed to air-dry. Afterward, the dye-bound cells were solubilized using 200µL of absolute ethanol, and the results were read using an ELISA reader at a wavelength of 630 nm.

Results

Bacterial isolates

Out of a total of 100 samples, 57 samples exhibited growth for gram negative bacteria ,collected from female 34 (59.64%) and 23 (40.36%) were from male patients in different age as table 2, these samples include 57 (57%) bacterial isolates contain 34 isolates of *E.coli* (59.64) % *Klebsiella pneumoniae* 17 (29.82%), *Pseudomonas aeruginosa* 4(7.01%), and Proteus mirabilis 2(3.5%) isolates.

Table 2.Distribution urine culture according to the sex

Patient	Number (%)
Female	34 (59.64%)
male	23 (40.36%)

Antibiotics Resistance profile

Bacterial isolates examined in this study showed resistance to tested antibiotics (Table 3).

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Table 3.Antibiotics Resistance of Uropathogens bacteria

Bacterial Isolates	Antibiotics						
	F	CIP	MEM	FEP	SXT	CN	AK
E.coli (34)	7(17.64)%	17(50)%	5(14.7)%	18(52.94)%	26(76.47)%	8 (23.52)%	14(41.17)%
K. pneumonia (17)	11(64.70)%	6(35.29)%	3(17.63)%	8(47.05)	13(76.47)%	3(70.58)%	7(41.17)%
P. aeruginosia (4)	2(50)%	3(75)%	2(50)%	3(75)%	4(100)%	2(50)%	2(50)%
P. mirabilis (2)	1(50)%	0%	0%	1(50)%	2(100)%	0%	1(50)%

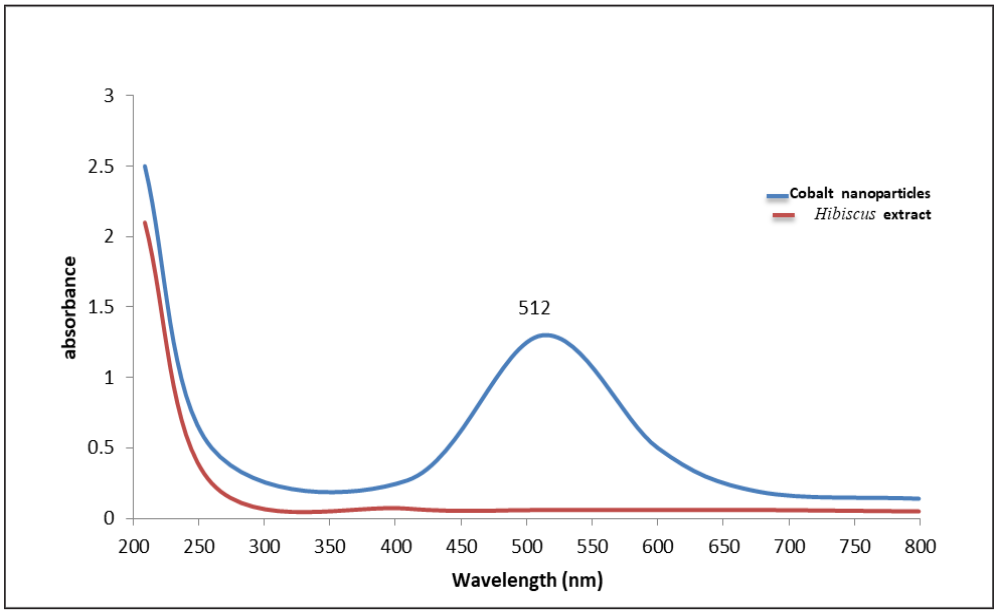

Figure 1.UV-Visible absorption spectra of synthesized Bio-Cobalt oxide nanoparticles

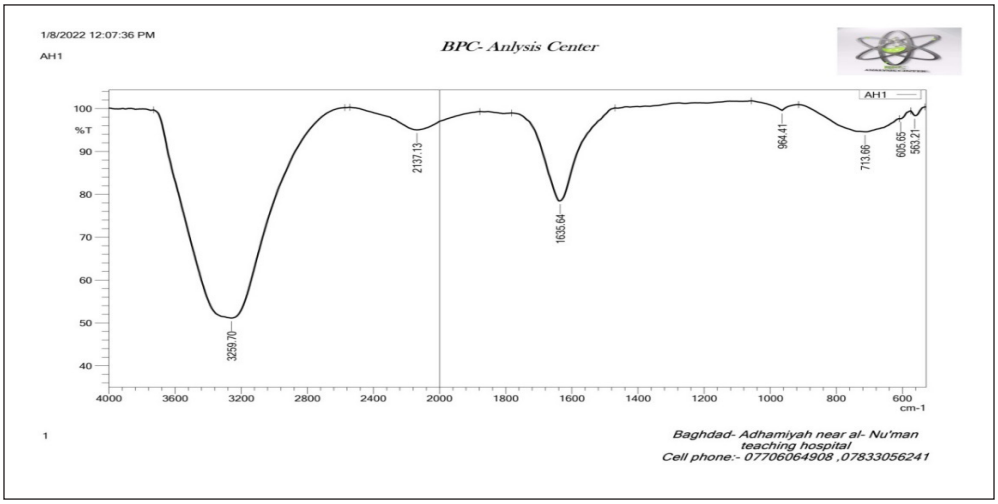

Figure 2.FTIR analysis of synthesized Bio-cobalt oxide nanoparticles

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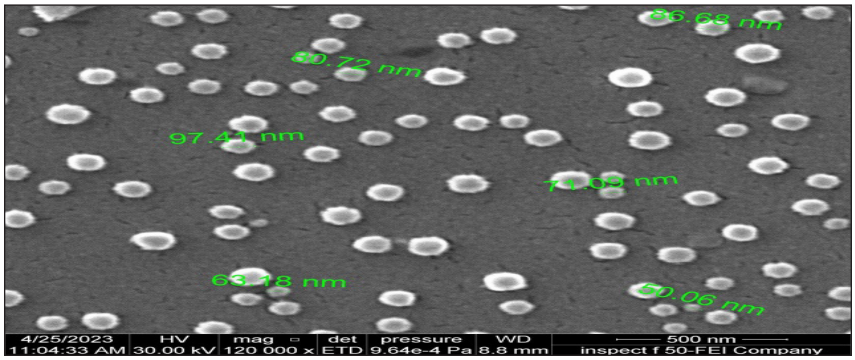

Figure 3.Scan Electron Microscopic (SEM) Image of synthesized Bio- Cobalt oxide nanoparticles

Cobalt oxide Nanoparticles Synthesis

The results of the ultraviolet spectroscopy examination showed an absorption peak at a wavelength of 512 nm (Figure 1).

The FTIR analysis identified bands at 563.21, 605.65, 713.66, 964.41, 1635.64, 2137.13, and 3259.70 cm^{-1}, as shown in Fig. 2.

The morphology and size of the cobalt oxide nanoparticles were determined by SEM examination, which showed that the particles were spherical in shape and had sizes ranging from 50.06 to 97.41 nm (Figure 3).

Antibacterial Activity of Cobalt oxide Nanoparticles

The cobalt oxide nanoparticle concentrations showed inhibitory activity against multidrug-resistant bacterial isolates, ten bacterial isolates which showed absolute resistance 100% to all seven antibiotics types compare with other bacterial isolates not appeared absolute resistance to same antibiotics For this reason, ten bacterial isolates were selected to determine the effect of cobalt oxide nanoparticles on them.

The results showed that the inhibitory effect of various concentrations of cobalt oxide nanoparticles affected on the bacterial isolates ten isolates in this study included five of *E. coli*, three isolates of *K. pneumoniae* and two isolate *P. aeruginosa* , all bacterial isolates exhibited resistance to the antibiotics in this study compare with concentration of cobalt oxide nanoparticles increased zone inhibition with increasing nanoparticle concentration (Table 4 and Figure 5).

Bacterial Biofilm formation and Antibiofilm Activity of Cobalt oxide Nanoparticles

The results showed that 10 (17.54%) bacterial isolates produced strong biofilm, 28 (49.12%) bacterial isolates produced weak biofilm, and 19(33.33%) did not produce biofilm. Therefore 10 isolates which produced strong biofilm were selected to evaluate the effect of cobalt oxide nanoparticles on biofilm formation, therefore only bacterial isolates produce biofilm very strong (ten isolates) selected to detect effect cobalt nanoparticles concentrations on bacterial biofilm

Table 4.The antibacterial effect (inhibition zone in mm) of synthesized Bio-cobalt oxide nanoparticles

Bacteria	CO-NPs				Antibiotics						
	25%	50%	75%	100%	AMC	CRO	CIP	CFM	AK	SXT	P
E. coli1	22	23	25.5	28	R	R	R	R	R	R	R
E. coli2	25	28	30	32	R	R	R	R	R	R	R
E. coli 3	18.5	22	24.5	27	R	R	R	R	R	R	R
E. coli 4	20	23	27	29	R	R	R	R	R	R	R
E. coli 5	22	24.5	26	28	R	R	R	R	R	R	R
K. pneumoniae1	18	21.5	24.5	26	R	R	R	R	R	R	R
K. pneumoniae2	19	22	24	25	R	R	R	R	R	R	R
K. pneumonia 3	16.5	20	23	26	R	R	R	R	R	R	R
Ps. aeruginosa1	17.5	21	23	24	R	R	R	R	R	R	R
Ps. aeruginosa2	17	20	22	23	R	R	R	R	R	R	R

*R= resistance

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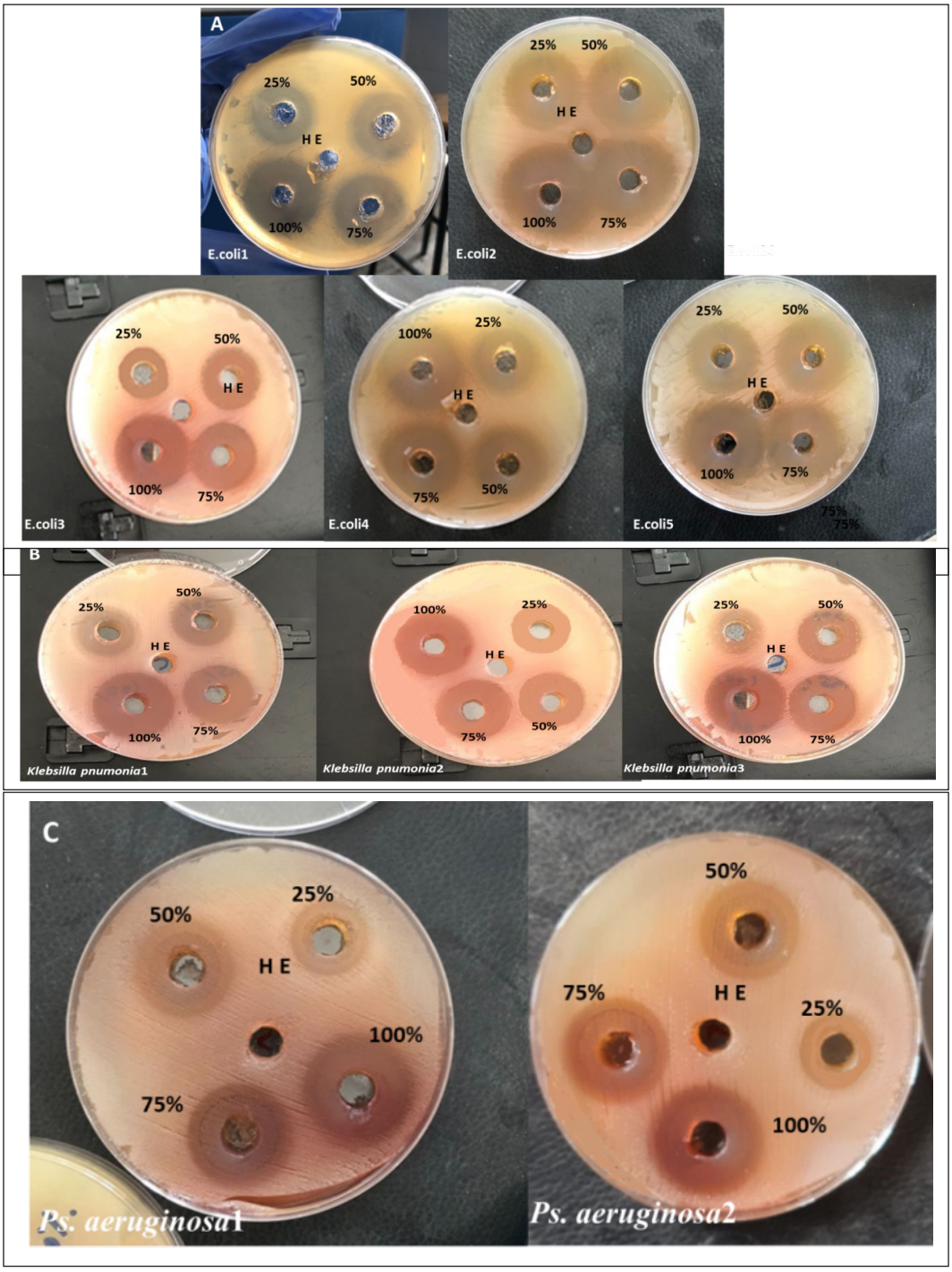

Figure 5.Antibacterial Effect (inhibition zone in mm) of Synthesized Bio-cobalt oxide nanoparticles (100,75,50,25)%, H. E. = Hibiscus extract (a)-E. coli, (b)- Klebsiella pneumonia, (c)- P. aeruginosa, Proteus

Congo Red Agar Method

The bacterial isolates formed black colonies on Congo red agar. However, after treatment with SubMic concentrations (25%, 50%,75%) of Cobalt oxide Nanoparticles , the colonies turned red (Figure 6).

Microtiter plate method

The results showed biofilm formation across all 10 bacterial isolates with absorbance values between 0.412 and 640 AU before treatment with Cobalt oxide nanoparticles. After treatment with Cobalt oxide nanoparticles, the absorbance values decreased to a range of 0.111-0.059 UV (Table 4).

Discussion

A urinary tract infection is most common infection of human , It may cause any part of urinary system and most common cause of these infection was Bacteria especially bacteria E. coli [27]

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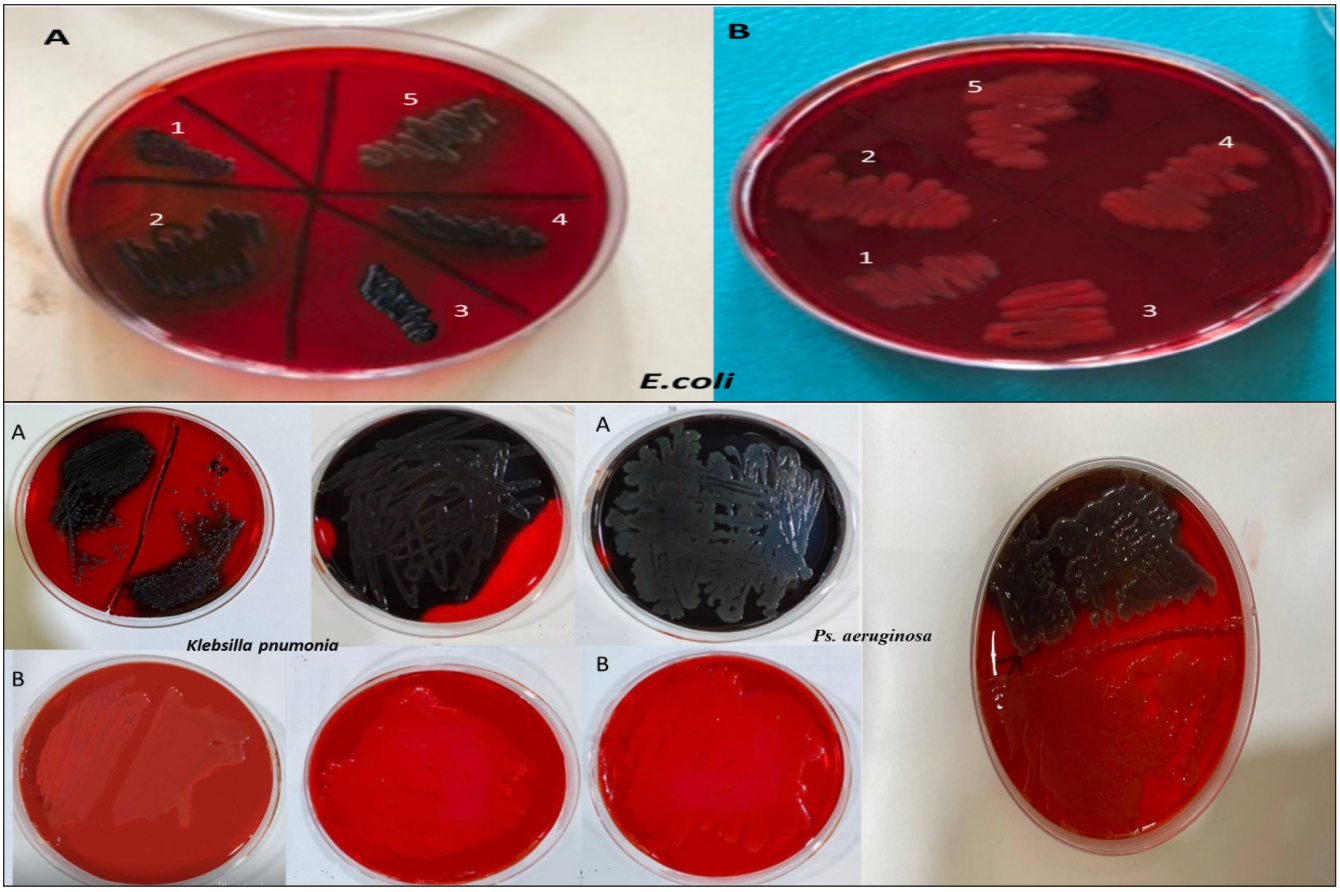

Figure 6.Bacterial Biofilm and Antibiofilm of Cobalt oxide nanoparticles on bacterial isolates. A before B- after treatment with Cobalt oxide nanoparticles

Table 4.Biofilm value formation before and after treatment with Cobalt oxide nanoparticles

NO.	Bacterial Isolates	Before treatment		After treatment	
		OD at 630nm	Biofilms	OD at 630nm	Biofilms
1	*E. coli 1*	0.455	Strong	0.077	Weak
2	*E. coli 2*	0.412	Strong	0.059	Weak
3	*E. coli 3*	0.492	Strong	0.099	Weak
4	*E. coli4*	0.521	Strong	0.101	Weak
5	*E. coli 5*	0.534	Strong	0.109	Weak
6	*K. pneumoniae1*	0.523	Strong	0.107	Weak
7	*K. pneumoniae2*	0.564	Strong	0.091	Weak
8	*K. pneumonia3*	0.572	Strong	0.107	Weak
9	*P. aeruginosa1*	0.640	Strong	0.102	Weak
10	*P. aeruginosa2*	0.636	Strong	0.111	Weak

This study found that *Escherichia coli* (*E. coli*) was one of the most common causes of urinary tract infections (UTIs), accounting for 34% of cases, and The percentage of females infected with gram negative bacteria (59.64%)was higher than males (40.36%). These findings are similar to those by [27], who found the prevalence of *E. coli* to be 37.7%, followed by *Klebsiella pneumoniae* at 11.4%, and Proteus at 1.6%. and females infectected by UTI infections 54.8% compare with male 45.2% Also [28] found that *E. coli* constituted 64.7% of the isolated samples from patients with UTIs, followed by *Klebsiella pneumoniae* (15.0%).

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This epidemiological pattern can be linked to anatomical, hormonal, and behavioral factors.[29] Urinary tract infections can be linked to the overuse of antibiotics, which affects the bacterial environment and contributes to the spread of antibiotic resistance. Multidrug-resistant *E. coli* (MDR-EC) strains have emerged as a clinical problem in recent decades, particularly affecting women with recurrent UTIs. The increasing incidence of MDR-EC, especially in developing countries, has led to the overuse of broad-spectrum antibiotics such as cephalosporins, fluoroquinolones, and aminoglycosides, resulting in higher treatment and hospitalization costs .[28, 30] The results showed that E.coli was highly resistant to SXT while resistance to meropenem was low. Similar results were obtained for *K. pneumoniae*. These findings are in line with those by [31] Refer to *E.coli* isolates showed 55.6%, 76.5% and 24% resistance to ciprofloxacin, trimethoprim-Sulfamethoxazole and Amikacin, respectively while Klebsiella pneumoniae showed resistance rates of 38.5 %, 83.3%, and 45.61% for ciprofloxacin, trimethoprim-Sulfamethoxazole and Amikacin, respectively.

The results showed that *Pseudomonas aeruginosa* exhibited resistance rates between 50% and 75% for the tested antibiotics, except for SXT to which there was 100% resistance. This is consistent with what has been reported by[32] In their study, the resistance rates of *Pseudomonas aeruginosa* isolated from urine samples were as follows: Nitrofurantoin, 98.89%; CIP, 30%; Meropenem, 43.70%; and Cefepime, 53%.[33] investigated the antimicrobial resistance status of *Pseudomonas aeruginosa* strains and found lowest susceptibility rates among the antibiotics studied were 62.2% for imipenem, 61.4% for meropenem, 57.7% for cefepime, and 66% for ciprofloxacin.[34] reported resistance rates of 42.3% for ciprofloxacin, 57.7% for cefepime, 29.6% for meropenem, and 50% for nitrofurantoin. Also [35] found resistance *P. aeruginosa* bacteria the antibiotics include Nitrofurantoin 81.8%, Ciprofloxacin (54.5%) and Amikacin (63%). Meropenem 45.5%, Trimethoprim/Sulfamethoxazole 100%, Gentamicin 63.6%, While E.coli showed resistance to antibiotic Nitrofurantoin 18.2% Meropenem 3.7% Ciprofloxacin 65.2%, Trimethoprim/Sulfamethoxazole 67.1%, and Gentamicin 37.3%

Also [27] detected resistance in *P. aeruginosa* to ciprofloxacin (50%) and trimethoprim- Sulfamethoxazole (60%). Results showed that broad-spectrum antibiotics, such as imipenem, meropenem and nitrofurantoin would be the first line and the most effective as the empirical treatment of urinary tract pathogens due to their low resistance rates, in comparison with other antibiotics which show high resistance like Ampicillin, Cefotaxime, Ceftazidime, Trimethoprim-sulfamethoxazole.[36] Bacteria have different mechanisms to antibiotics resistant Such aminoglycoside group antibiotics as Amikacin and Gentamicin bacteria résistance to these antibiotics through production aminoglycoside -modifying enzymes lead to destroyed antibiotic by analysis amides and esters bonds found in structure of aminoglycosides antibiotics . [28] while bacteria resistance to β-lactam antibiotics as Cefipem by Modifications of penicillin binding proteins (PBPs) site on cell wall of bacteria was target to β-lactam antibiotics is modification lead to resistance bacteria to β-lactam antibiotics , Bacteria reduce affinity to PBPs and increase expression of β-lactamase which lead to hydrolysis of β-lactam ring of antibiotics . [37]

While resistance bacteria to antibiotics fluoroquinolone group such as antibiotic Ciprofloxacin due to mutations in bacterial DNA encoding gyrase and topoisomerase enzymes lead to destroyed these antibiotics [38], another mechanism includes modification of bacterial metabolic pathways such Trimethoprim-sulfamethoxazole antibiotic bacteria resistance to this antibiotic through through absence or alteration of para-aminobenzoic acid, was a precursor to nucleic acid and synthesis folic acid, lead to allows bacteria to overcome inhibitory effects of antibiotic targeting these pathways .[39]

The chemical or physical methods used to synthesize nanoparticles are associated with several drawbacks, including the production of toxic chemicals harmful to humans and environmental pollutants. Additionally, these processes are expensive, require high energy consumption, and are time consuming. To overcome these problems, the green synthesis methods for producing nanoparticles are being preferred due to their simplicity, environmental friendliness, and cost-effectiveness. [7,40] The process of producing nanoparticles via the green method uses various sources, including plants, bacteria, algae, and fungi .[41] Plants have been extensively utilized to synthesize cobalt oxide nanoparticles because they are readily available, easy to access, safe to use, and facilitate the reduction and stabilization of nanoparticles through phytochemicals .[42] In this study, an aqueous extract of the hibiscus plant was employed as a reducing and stabilizing agent for the production of cobalt oxide nanoparticles. The synthesis of cobalt oxide nanoparticles was indicated by a color change in the reaction mixture from light red to reddish brown, consistent with the findings reported by [43] To verify the formation of these nanoparticles, UV spectroscopy was conducted, revealing a peak absorbance at 512 nm, attributed to the surface plasmon resonance absorption of the cobalt oxide nanoparticles. This phenomenon arises from the confined collective oscillation of electrons in the conduction band, which is stimulated by incoming electromagnetic radiation. The observed color change in the reaction mixture is also related to this surface plasmon resonance effect, serving as a reliable indicator for the formation of cobalt oxide nanoparticles .[44]

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The FTIR analysis identified biomolecules that play a role in the reduction and capping of the cobalt oxide nanoparticles. It revealed diverse absorption bands for the cobalt oxide nanoparticles, including 563.21, 605.65, 713.66, 964.41, 1635.64, 2137.13, and 3259.70 cm^{-1}. These results are close to the findings of [20], who reported bands at 1350, 1634, and 3293 cm^{-1} in the FTIR analysis of cobalt oxide nanoparticles synthesized from hibiscus.[45] showed that bands at 566 and 667 cm^{-1} indicated the presence of Co–O and O–Co–O groups respectively which confirmed the formation of cobalt oxide nanoparticles. The absorption peak at 3228 cm^{-1} corresponds to the O–H stretching vibrations of hydroxyl groups .[46] The band at 1644 cm^{-1} which represents the C=O stretching, indicates the presence of carbonyl groups in carboxylic acids, ketones and esters [47]). Furthermore, FTIR analysis confirms the successful coating of plant metabolites on the surface of cobalt oxide nanoparticles. These results suggest that the presence of phenolic compounds and proteins was responsible for the formation and stability of the synthesized cobalt oxide nanoparticles, [11]. In the current study the particle sizes determined by SEM ranged from 50.06 to 97.41 nm, which are consistent with the results reported by [48] who noted that the cobalt oxide particles with sizes determined by SEM were greater than 50 nm. Additionally, showed that the average size of the cobalt oxide particles ranged from 40 to 60 nm by SEM.49

Our study demonstrated that the bacterial isolates had the ability to produce biofilms, as assessed using both the Congo red plate and the microtiter plate methods. This study near to results [50] who observed in their experiment that among 37 isolated E. coli strains, 16 (43.2%) were strong biofilm producers, indicating the role of these virulence factors in urinary tract infections (UTIs). Also [51] found that all isolates of *K. pneumoniae* produced biofilms; specifically, seven isolates were strong biofilm producers according to tests using Congo red plates and microtiter plates, corroborating our findings regarding biofilm formation in *K. pneumoniae* isolates. Furthermore [52] reported that a higher proportion (44 or 89.8%) of the bacteria *Pseudomonas aeruginosa* were identified as biofilm-forming using the microtiter plate method.

The results of the current study indicate that cobalt oxide nanoparticles synthesized using hibiscus extract possess effective antibacterial activity, with inhibitory zone diameters ranging from 23 to 32mm against isolates of *E. coli, P. aeruginosa*, and *K. pneumoniae*. These values are close to the results reported by [20] who demonstrated the antibacterial effectiveness of cobalt oxide nanoparticles synthesized by the green method using hibiscus leaf extract against *E. coli* with an inhibition zone of 28 mm. Furthermore, [53] reported inhibition zones of cobalt oxide nanoparticles using the well diffusion method against *E. coli* (18 mm), *K. pneumoniae* (19.5 mm), and P. aeruginosa (20 mm). Additionally, [49] found that cobalt oxide nanoparticles demonstrated inhibition using the well diffusion method against *E. coli* with an inhibition zone of 29 mm. Bacteria may carry negative charges on their surface due to lipopolysaccharide (LPS) compounds present in their cell walls, which enables them to interact with positively charged nanoparticles. [54] Ions derived from positively charged nanoparticles are attracted to the surface of Gram-negative bacteria due to the presence of lipopolysaccharide [55]Furthermore, the cobalt oxide nanoparticles may directly interact with the outer membrane of bacteria, leading to their aggregation on the membrane and altering its physical and chemical properties , which may result in its disruption, thus killing the bacteria [56] The antibacterial activity of the nanoparticles is attributed to their high surface-area-to-volume ratio, which allows them to interact closely with microbial membranes thereby increasing their biological and chemical effectiveness. [54,55] Furthermore, due to their small size the nanoparticles can penetrate the bacterial cell membrane and inhibit bacterial growth [55] The generation of free radicals such as hydrogen peroxide, in the presence of nanoparticle ions, leads to damage to genetic material and disruption of protein function. In particular, cobalt oxide ions interfere with the thiol groups of bacterial enzymes, resulting in cell damage and bacterial death. [57]The concentrations of cobalt oxide nanoparticles appeared to inhibit the biofilm formation of bacterial isolates. This finding is consistent with the study by [58] who reported the effect of cobalt oxide nanoparticles on biofilm formation in *E. coli* associated with urinary tract infections. [59] demonstrated that cobalt oxide nanoparticles at a concentration of 10 mg/mL resulted in a maximum biofilm suppression activity of 76.54%, while a lower concentration of 0.156 mg/mL showed only 11.50% suppression, as assessed using the microtiter plate method. [60] evaluated the anti-biofilm activity of cobalt oxide nanoparticles at a concentration of 80 µg/mL specifically against multidrug resistant (MDR) *E. coli*. Furthermore [61] found that gold nanoparticles (gNPs) led to a decrease in the expression levels of virulence factors and biofilm production. Similarly [62] showed that these nanoparticles inhibited the production of virulence factors, including biofilm formation in *K. pneumoniae*. We need more extensive molecular profiling and future investigations with larger cohorts. The diagnosis of UTIs and other medical disorders are greatly aided by molecular and immunological methods, which have been applied in many studies [63-70]

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Despite the diverse chemical, biological, and physical properties of cobalt nanoparticles, it is essential to investigate their potential toxicity. Previous studies have indicated that cobalt nanoparticles can induce cellular stress—specifically through the generation of reactive oxygen species (ROS) or oxidative stress—and trigger inflammatory responses. High concentrations may lead to genetic or cellular damage. The toxic effects of these nanoparticles depend on several factors, including concentration, particle size, surface properties, and the release of cobalt ions. Therefore, dose-dependent studies on cytotoxicity and genotoxicity are necessary to assess the material's viability for practical use before considering broader biomedical or environmental applications, while simultaneously interpreting the observed biological activity in the context of safety concerns. [71,72]

Conclusions and Recommendations

This study presents a novel method for synthesizing cobalt oxide nanoparticles using hibiscus petal extract, offering a cost-effective and eco-friendly solution against multidrug-resistant Gram-negative bacteria. The synthesized nanoparticles showed significant antibacterial activity against clinical isolates of *E. coli*, K. *pneumoniae*, *P. aeruginosa* and *Proteus* mirabilis with inhibition zones ranging from 23 to 30 mm. This approach contrasts with previous studies which focused on chemical synthesis methods. The study highlights the potential of bio-synthesized cobalt oxide nanoparticles as alternatives to traditional antibiotics. Future research should explore their antibacterial mechanisms and clinical applications to combat antibiotic resistance. Integrating these nanoparticles into treatment protocols could lower healthcare costs, improve treatment outcomes, and support sustainable practices in Nano medicine, benefiting both the economy and the environment.

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