

*Microbial Science Archives* (ISSN: 2583-1666)

An International Research Journal

Journal homepage: [www.microbial.sciencearchives.org](http://www.microbial.sciencearchives.org)

<https://doi.org/10.47587/MSA.2023.3105>



Research Article

## Biosynthesis of cobalt oxide nanoparticles using blood serum as reducing and stabilizing agent for therapeutic use against multi-drug resistant *Acinetobacter baumannii*

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Received: Feb 21, 2023/ Revised: Mar 10, 2023/Accepted: Mar 14, 2023

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### Abstract

Multi-drug resistant bacteria *Acinetobacter baumannii* developed as a result of improperly used antibiotics, which are hard to treat and led to serious health problems all over the world. Therefore, it has become necessary to find some alternative ways to overcome this problem and secure human health. In this study, cobalt oxide nanoparticles were biosynthesized using blood serum from individuals with high blood levels as a reducing and stabilizing agent. Additionally, research on the therapeutic uses of the created nanoparticles' antibacterial activity against the human pathogenic bacteria *A. baumannii* is being done. Co oxide NPs formation was characterized and confirmed using UV-VIS, atomic force microscopy (AFM), X-ray diffractometer (XRD), and transmission electron microscopy (TEM). Biosynthesized Cobalt oxide NPs appeared in polycrystalline structure with an average diameter of 43.45nm. Bacterial isolates were identified using morphological, gram stains, and biochemical tests for more confirmation Vitek-2 system was used also and the results showed that out of 172 clinical samples, 91 isolates were identified as *A. baumannii*. Antibiotic Susceptibility test was done against 12 various antibiotics to determine the MDR isolate for further study. Anti-bacterial activity test against MDR *A. baumannii* was carried out using different concentrations of Co oxide NPs (1.25, 2.5, 5, 10, and 20 mg/mL). The biological activity results of biosynthesized Co oxide NPs showed that the maximum inhibition concentration was 20mg/ml with a zone diameter of 22 mm while the minimum inhibition concentration (MIC) was 5 mg/ml with a 10mm zone diameter.

**Keywords:** Cobalt Oxide, Nanoparticle Characterizations, Biosynthesis, Serum, *A. baumannii*, Antibacterial Activity

### Introduction

Nanotechnology is a neoteric branch of technology that is concerned with the utilizing of macromolecule materials at the nanoscale sizes, in other words, nanotechnology is a recent field of science that deals with and manipulates materials, especially atoms and outputting three-dimensional, high surface area nanoparticles ranging between 1-100 nanometers (Abass et al., 2021). Nanotechnology extraordinarily has participated to improve and progressing a wide variety of fields of science such as physics, chemistry, biotechnology, medicine, and other fields related to our life (Abdal et al., 2017). To date various forms of nanoparticles have been produced, differing in size, shape, surface area, and purpose of

use, concerning the biological fields; nanoparticles are divided into three groups; inorganic nanoparticles, organic nanoparticles, and hybrid nanoparticles. As a result of their unique properties, Metal and metal oxide nanoparticles (inorganic nanoparticles) are playing a major role in combating microbial communities they are considered antibacterial and anti-biofilm agents (Ajarem et al., 2022; Al-Abdulla, 2012; Al-Haideri, 2019).

Cobalt oxide NPs are widely used in a variety of everyday applications due to their high resistance to oxidation as well as corrosion. Cobalt nanoparticles (NPs) have been generated utilizing many physicochemical procedures, such as pyrolysis, hydrothermal decomposition, modified polyol approaches,

solvothermal liquid-phase reduction, and template-based processing techniques. These methods have many drawbacks, including energy and time-intensive processes, the need for specialist equipment, and environmental harm. To address energy imbalances and toxic wastes, some greener and eco-accommodating methods have been proposed. These methods are simple, non-hazardous, and cost-effective (Mohammadinejad, et al., 2019; Verma, et al., 2016). Organisms can synthesize cobalt and cobalt oxide nanoparticles in one process, making them environmentally beneficial. Furthermore, biomolecules from these organisms, such as steroids, enzymes, amino acids, proteins, saponins, tannins, proteins, phenols, vitamins, sugars, and flavonoids, the majority of them are of biomedical and pharmaceutical importance and are eco-friendly (Iravani and Varma 2020). The serum is a transparent liquid that resembles blood plasma but lacks clotting factors. The serum contains water, proteins, carbohydrates, glycerol, and fatty acids. It contains hormones, vitamins, enzymes, colors, and cellular waste such as uric acid, and amino acids. These components vary widely between people. This distinction extends to the same person and occasionally to others (Al-Abdulla, 2012). *A. baumannii* is a strictly aerobic, gram-negative, non-fermenting coccobacillus. These bacteria had previously been regarded as a low-level pathogen despite its ability to cause numerous and serious infections, including those of the skin, circulation, urinary tract, secondary meningitis, and soft tissues.

## Methods and Materials

### Bacterial collection, isolation, and culture media

The Martyr Ghazi Al-Hareery Hospital for Surgical Specialties (Baghdad Medical City), Samarra General Hospital, Burns Specialty Hospital (Baghdad Medical city), and Child Care Hospital were the four Iraqi hospitals from which 172 clinical specimens, including wounds, burns, urine, and sputum, were collected in sterilized containers. The collected samples were streaked immediately on blood agar and MacConkey agar in the aseptic laboratory of each hospital, where they were subsequently incubated for 24 hours at 37°C. This media was prepared on blood agar under the manufacturer's instructions. Depending on the results of morphological characteristics and biochemical tests, identification tests.

### Antibiotic Susceptibility of *Acinetobacter baumannii*

The bacterial suspension with  $1.5 \times 10^8$  CFU/ml (McFarland) was transferred into Mueller-Hinton agar (Effendi and Harijani, 2017) using a sterile cotton swab. It was then carefully spread out and allowed to sit for 10 minutes as part of the antibiotic sensitivity test. The susceptibility test was performed using 12 antibiotic discs: azithromycin (15 mg), cefotaxime (30 mg), colistin (10 mg), ciprofloxacin (10 mg), doxycycline (10 mg), imipenem (10 mg), meropenem (10 mg), methicillin (10 mg), nitrofurantoin (100 mg), tobramycin (10

mg), trimethoprim-sulfamethoxazole (25 mg), and vancomycin (30 mg). The plates were then turned over and left to sit at 37 degrees for 18 to 24 hours. The inhibition zones around the discs were measured using millimeters (mm) of a metric ruler, per the Clinical Laboratories Standards Institute. According to the standard inhibition zones of antibiotics, each isolate was divided into three categories: those that were sensitive (S), intermediate (I), and resistant (R) to a particular drug. For further research, multidrug-resistant *A. baumannii* was chosen using this test.

### VITEK 2 System Identification

For more confirmation the most susceptible to antibiotic isolate of *Acinetobacter baumannii* was grown overnight on a Brain heart infusion agar plate to be identified by VITEK 2 system (Biomérieux, France). This system's Gram-negative (GN) card is used to automatically identify the most significant fermenting and non-fermenting Gram-negative bacterial species (ASM 1998).

### Collection and preparation of blood serum

In the blood bank at Aldhuluiya General Hospital, Salahuddin, Iraq. Blood serum is drawn from young males (25–40 years) suffering from high blood levels. Individual donor units of whole blood are drawn using an industry-standard empty blood pack with an integral donor tube. 15-gauge needle blood is then transferred directly to a gel tube after fully discharging of EDTA solution. Gel tubes are then centrifuged at 2000 rpm for 10 minutes, after which the clear serum layer is aseptically transferred to a new 250-mL container with appropriate inert cap. Serum units are stored at 2 °C to -8 °C under dark conditions (Danilenko et al., 2020).

### Biosynthesis of Cobalt Oxide Nanoparticles

Cobalt oxide NPs were prepared using blood human serum and this is the first time, depending upon (Siddique et al., 2021) with some modifications as follows: 10 gm of cobalt chloride hexahydrate ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) obtained from HIMEDIA, India) was used as precursors added into 100 ml of serum solution, mixed well, serum color changed into violet, overnight shaking at 150 rpm at room temperature with pH 6.9, under dark conditions. The next day solution was centrifugation at 8000 rpm for 30 minutes' supernatant was discharged while the precipitant was washed again with Deionized Water, centrifuged at 8000rpm for 30 minutes for a second-time precipitant was collected in a glass petri dish then stored 24 hours in an incubator at 40°C for next work characterization and medical application.

### Characterization of Cobalt oxide NPs

Cobalt oxide Nanoparticles were characterized by several techniques like UV- visible, AFM, XRD, and TEM. UV-visible was done by using (Shimadzu UV-1800- Vis

Spectrophotometer, AFM (Atomic force microscopy) was used to find out the average diameter of cobalt Nanoparticles, XRD (X-ray Diffraction) analysis was also used to know the element identity, the prepared cobalt oxide nanoparticles were morphologically analyzed by TEM (Transmission electron microscopy).

### Antibacterial activity assay

The agar well diffusion test was applied to determine the antibacterial activity of the Green-prepared Co oxide NPs. Selected MDR isolates of *A. baumannii* were activated in BHIB for 18- 24 hours. The inoculum was prepared at McFarland 0.5 OD 630 ( $1.5 \times 10^8$  CFU/ml). The overnight growing bacteria was transferred and spread onto the agar medium by using sterile cotton swabs, then (4mm) wells were made using yellow tips. Different Co oxide NPs concentrations (1.25, 2.5, 5,10, and 20 mg/mL) were prepared separately and used in this test. Overnight incubation and then the anti-microbial activity of Co oxide NPs determined by measuring the inhibition zone around the wells (Altaee, et al., 2020, Seil and Webster, 2012).

## Results and Discussion

### Isolation and Identification of *Acinetobacter baumannii*

The results of microscopic examination (Gram Staining) and biochemical tests were used to identify *A.baumannii* isolates showed that out of 172 clinical specimens of wounds, urine, burns, and sputum, 91 isolates were identified as *A. baumannii* and 81 isolates from another bacterium. These positive isolates were obtained in high percentages; 42.86% (n=39) from wound specimens; urine specimens constituted 27.47% (n=25), while the percentage of burn specimens was 21.97% (n=20), and the low percentage was obtained from sputum specimens. Which achieved 7.70% (n=7), Number and percentage of *A. baumannii* isolates per specimens' source were shown in Table 1.

**Table 1. Number and percentage of *Acinetobacter baumannii* isolate in accordance to specimen source**

Source of specimens	No. of samples (%)	No. of <i>A. baumannii</i> (%)
Wound infection (swab)	61 (35.47%)	39 (42.86 %)
Urinary tract infection	52 (30.23%)	25 (27.47%)
Burn infection (swab)	41 (23.84%)	20 (21.97%)
Respiratory tract infection	18 (10.46%)	7 (7.70%)

### Antibiotic Susceptibility of *Acinetobacter baumannii*

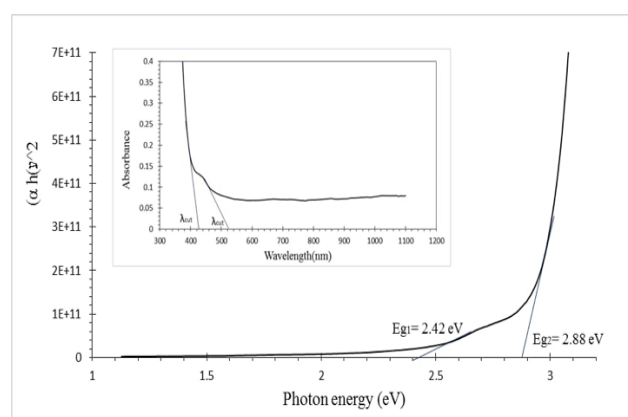
This study concluded that a total of 91 *A. baumannii* clinical isolates had a high resistance to both Methicillin (85.71%) and Nitrofurantoin (81.32%). This study also exhibited high resistance to Vancomycin (79.12%), Azithromycin (74.735%), Cefotaxime (61.54%), Imipenem (57.14%), Tobramycin (56.05%), Meropenem (53.84%), Trimethoprim sulfamethaxazole (51.65%). Ciprofloxacin (47.25%) and Doxycyclin (36.26%) showed moderate resistance, while Colistin showed low-level resistance (17.58%). The differences in the susceptibility patterns may be due to the type of patients enlisted for the study and the source of collected specimens. This test was used to select a multi-drug resistance *A. baumannii* for further study.

### VITEK 2 System Identification

VITEK 2 compact system was carried out as a confirmatory test for the identification of the multi-drug resistance, *A. baumannii*, and the characterization of the results for *A. baumannii* by using the VITEK2 compact system shown in Table 2.

### UV-VIS spectral analysis Co oxide NPs

Materials' UV-visible spectra in aqueous solutions were recorded, and direct band gap energy was computed using the Tauc relation. The plot of  $(\alpha h\nu)^2$  versus photon energy 'h' reveals the nanoparticle's steep absorption edge. Eg was determined by extrapolating the plot's linear section, as seen in Figure 1. Two different optical transitions were noted for the chemically created materials Co<sub>3</sub>O<sub>4</sub> at 2.88 (Eg1) and 2.42 eV. (Eg2). In general, O<sub>2</sub> Co<sub>3</sub> + (Eg1) charge transfer is linked to the lower band gap transitions, and O<sub>2</sub> Co<sub>2</sub> + (Eg2) charge transfer is linked to the higher band gap transitions (Meher and Rao, 2011).



**Fig. 1 Optical properties of Co oxide NPs**

Table 2. Identification information of vitek2 test for *A. baumannii*

bioMérieux Customer: System #: قسطوره م		Laboratory Report				Printed by: Labadmin											
Card Type: GN Bar Code: 2411294113444326 Testing Instrument: 0000148FFC51 (9517) Setup Technologist: Laboratory Administrator(Labadmin)		Bionumber: 0041010101400350															
Organism		Selected Organism: <i>Acinetobacter baumannii</i>															
Comments:																	
Identification Information		Card: GN	Lot Number: 2411294113	Expires: Jun 12, 2021 13:00 CDT													
		Status: Final	Analysis Time: 9.92 hours	Completed: Aug 17, 2021 21:38 CDT													
Organism Origin		VITEK 2															
Selected Organism		Acinetobacter baumannii complex Bionumber: 0041010101400350 Confidence: Low discrimination															
Analysis Organisms and Tests to Separate:		Low Discrimination Organism Acinetobacter baumannii complex Acinetobacter nosocomialis B-HEM(1),GELATIN(1), Acinetobacter pittii 44C(1),B-HEM(1), Acinetobacter baumannii 44C(99),Arg.hydr.(99),B-HEM(1), Acinetobacter calcoaceticus Arg.hydr.(99),B-HEM(1),GELATIN(1), Acinetobacter haemolyticus Arg.hydr.(1),B-HEM(99),GELATIN(96).															
Analysis Messages:		Contraindicating Typical Biopattern(s) Acinetobacter baumannii complex dCEL(79).															
Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	SKG	-
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	-			-

Atomic Force Microscope (AFM)

Atomic force microscope (AFM) analysis was used to confirm the morphology and particle size of cobalt oxide NPs, and the results are displayed in (Table 3) and (Fig. 2). AFM was used to examine the surface shape development of Co oxide nanoparticles and show the 2D and 3D appearance of Co oxide nanoparticles (Vera et al., 2009). The average size of Co oxide nanoparticles, which was found to be 43.45 nm, was also ascertained using AFM. Co3O4 nanoplatelets with an average diameter of 40 to 80 nm have been seen in another study by (Bibi et al., 2017), and atomic force microscopy analysis revealed both spherical and pentagon-like cobalt oxide nanoparticles in the size range of 20 to 90 nm (Sundararaju et al., 2020).

Table 3. Average diameter of Co oxide NPs

Sample	Average diameter(nm)	Roughness (nm)	R.M.S (nm)
Co	43.45	18.84	23.16

X-Ray Diffractometer of cobalt oxide NPs

By comparing the Co oxide NP, diffraction angles (2θ) with the JCPDS card number in the literature, the x-ray diffraction (XRD) analysis was done to confirm the crystal structure and

phase purity of the Co oxide nanoparticles generated with the biosynthetic approach. For the Co NPs, the XRD pattern was captured at a 2° angle ranging from 10° to 80°, as shown in Fig. 3. The Co NPs' XRD pattern revealed diffraction

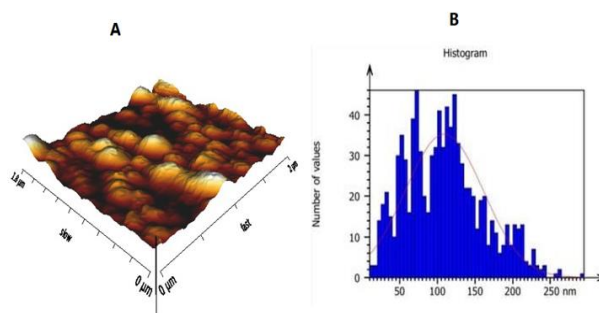


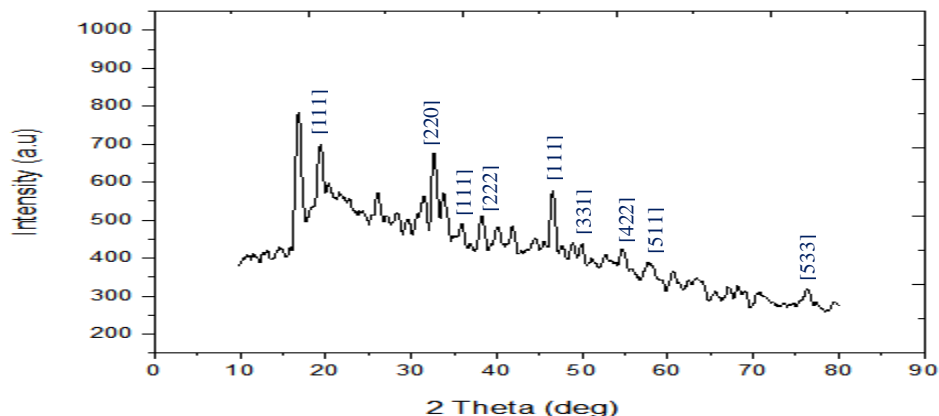
Fig. 2 Atomic force microscopy (AFM) of Co oxide NPs synthesized using human blood serum (A) high-measured trace 3D (B) Particle size distribution curve

peaks at angles of 18.95° and 38.55° that was related to the Co3O4 structure and indexed to the Miller indices of (111) and (222), as well as at angles of 32.45°, 49.54°, 55.45°, 58.64°, and 77.145° that were related to the Co3 structure and

indexed to the Miller indices of (220), (311), (4 111). The Co structure was connected to the diffraction peaks at  $46.51^\circ$  angles, which were linked to the Miller indices of (111). This complemented the (JCPDS Card No. 96-42-1467, 96-71-1178, and 96-02-0925). Face-centered cubic and densely packed hexagonal formations are both present in cobalt. The nanoparticle structure is polycrystalline, as was mentioned. The Debye-Scherrer equation is used to get the typical cobalt oxide granular size, as shown in Table 4.

**Table 4.** shows the average granular size of cobalt oxide using the Debye-Scherrer equation

FWHM	2THETA	C.S	hkl	sample
0.214	18.95	37.73369	[111]	Co <sub>3</sub> O <sub>4</sub>
0.381	32.45	21.76862	[220]	Co <sub>3</sub>
0.24	36.8	34.96647	[111]	CoO
0.1914	38.55	44.07269	[222]	Co <sub>3</sub> O <sub>4</sub>
0.34	46.51	25.48611	[111]	CO
0.38	49.54	23.07182	[311]	Co <sub>3</sub>
0.44	55.45	20.43562	[422]	Co <sub>3</sub>
0.51	58.64	17.89771	[511]	Co <sub>3</sub>
0.325	77.145	31.29778	[222]	Co <sub>3</sub>



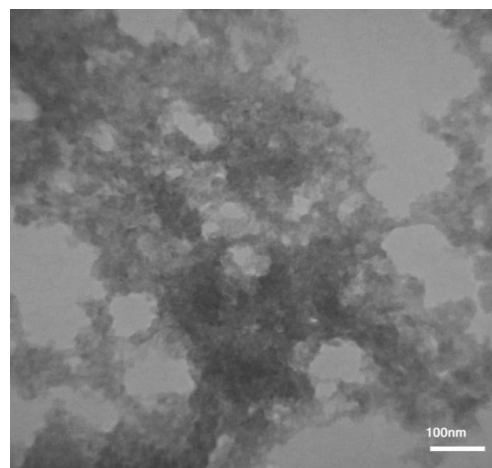
**Fig. 3** XRD pattern of Co oxide NPs synthesis by biosynthesis

Similar XRD patterns were also recorded in previous reports by (Urabe and Aziz, 2019) another Co oxide XRD result agreed with this study and was obtained by (Siddique et al., 2021). Very close results obtained by (Ajarem et al., 2022) they have shown that the high diffracted intensities were around  $2\theta = 33.2^\circ, 39.4^\circ, 44.8^\circ, 56.4^\circ, 59.5^\circ, 65.3^\circ,$  and  $77.2^\circ$  which are correlated to the Miller indices of (220), (311), (400), (422), (511), (440) and (533). Thus, indicates the formation of the crystalline phase of Co oxide NPs.

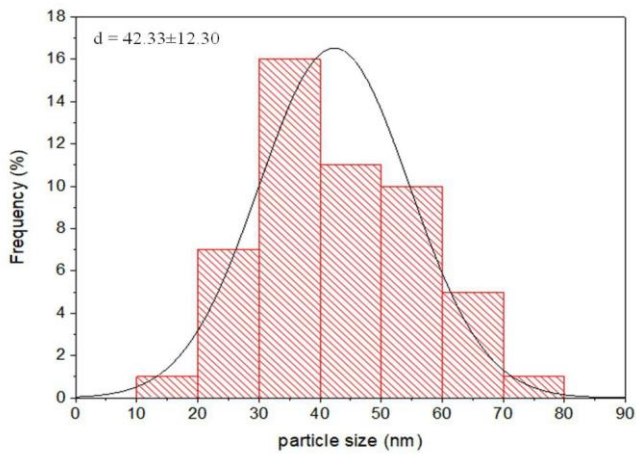
#### Transmission Electron Microscopy (TEM) analysis

At magnifications between 60 and 100 nanometers, transmission electron microscopy (TEM) investigation was done to verify the biogenesis of the Co oxide nanoparticles. TEM analysis was also used to gather data on shape, size, and aggregation. Transmission electron microscopy (TEM) measurements of Co<sub>3</sub>O<sub>4</sub> and Co oxide NPs revealed a size distribution of (from 10-80) nm on average. Both NPS were spherical and devoid of a porous structure, according to TEM pictures (Fig. 4). Both NPs displayed "hard agglomerates," according to the hydrodynamic size of the particles. The TEM picture was examined, and particle sizes were determined using the Image J software tool. The size distribution histogram shows that the average size of the Co oxide NPs

was 42.33 nm as indicated in the size distribution histogram in Fig. 5.



**Fig. 4** TEM images of Co oxide NPs synthesized by biosynthesis

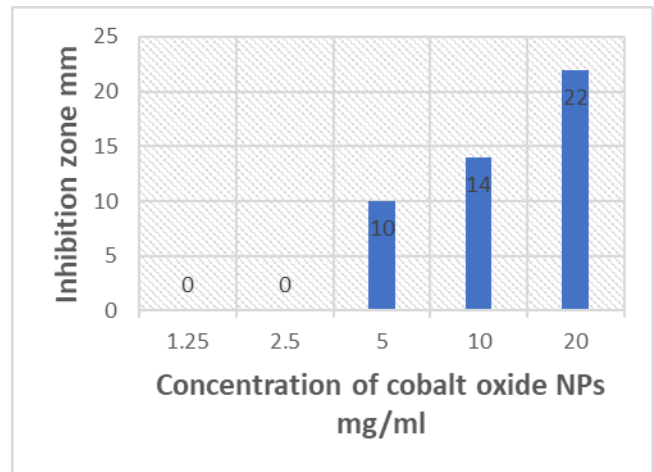


**Fig. 5** Size distribution histogram of Co oxide NPs

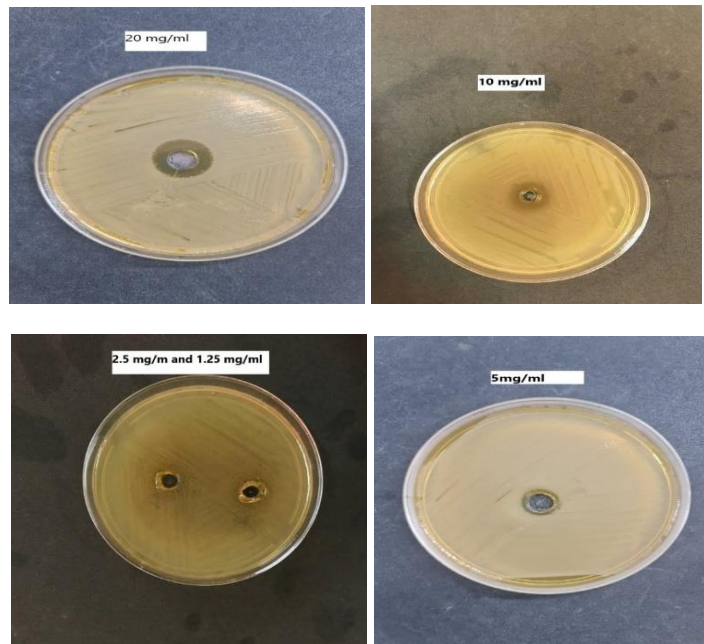
There are several studies to use TEM for cobalt oxide nanoparticles a study carried out by (Hafeez et al.,2020) they have been prepared  $\text{Co}_3\text{O}_4$  NPs using leaves extract of *Populus ciliata* (safaida) and they observed in the large area TEM image, that few nanoparticles are aggregated together in the form of groups, and also they estimated the NPs size in the range about 15-35nm. Nazeruddin et al., (2017) found that the aggregation of the NPs primarily depends upon the nature of the plant extract and the phytochemicals since these biomolecules cap and stabilize the individual particle.

**Antibacterial assay**

The maximum inhibition zones of *A. baumannii* were 22 mm at 20 mg/ml of cobalt oxide nanoparticles, while the minimum inhibition zones were 10 mm at 5 mg/ml. 2.5 and 1.25 mg/ml showed no zone, and the minimum inhibition concentration (MIC) of this study was 5 mg/ml (Fig. 6). In, Co oxide NPs showed antimicrobial activity (Fig. 7).



**Fig. 6** Antibacterial activity of cobalt oxide NPs against *A. baumannii*



**Fig. 7** Inhibition zone diameter of cobalt oxide NPs against *A. baumannii* NPs at different concentrations (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5mg/ml, and 1.25mg/ml)

Khan et al. (2015) found that biosynthetic cobalt oxide with an average diameter of 36 nm killed five Gram-negative bacteria isolates. They found that nanoparticle concentration affects inhibitory action. In another study (Hafeez et al., 2020), Co<sub>3</sub>O<sub>4</sub>'s maximum inhibition zone against gram-negative bacteria (*Klebsiella pneumonia*) was (20.4 0.7) with an average diameter of 40–50 nm. This study found that NPS size and Co oxide NPs concentration affect the antibacterial activity and that Co oxide NPs effect depends on the NPs characteristics, particle concentration, bacteria strains, and testing conditions. These findings are consistent with (Abass, et al., 2021; Khalil et al., 2020; Gupta et al., 2020; Hamza and Yaaqoob 2020; Kareem et al., 2019; Satpathy and Manikandan 2019; Anwar et al., 2019). The discrepancy in inhibition diameter may be due to NP-microbe interactions and the susceptibility of the bacteria used in the current study. Metallic nanoparticles' antibacterial mechanism is unclear. However, Co oxide NPs antibacterial actions might be summarized as follows: First, the extremely surface-to-volume ratio of small Co NPs changed the structure and permeability of the bacterial outer membrane. Greater cell permeability allowed NPS to enter. Co-NPs enter cells and change into an unstable ionic state, causing cellular oxidative stress and highly reactive oxygen species (Abdal Dayem et al., 2017). Nucleus disintegration and cell wall electron imbalance cause DNA damage (Kong et al., 2020). Co oxide NPs may interact with thiol groups (-SH) of bacteria-produced enzymes to inactivate and kill infections (Hafeez et al., 2020). Bacteria can carry metal ions due to protein thiol groups (-SH). The NPS inactivated proteins and nutrients entering the cell membrane, killing the bacterium (Irvani and Varma 2020).

## Conclusion

Biosynthesized Cobalt oxide NPs showed remarkable inhibition activity against the growth of *A. baumannii* it may be considered one of the most promising methods for treating these types of bacterial infections.

## Conflict of Interest

The author hereby declares no conflict of interest.

## Consent for publication

The author declares that the work has consent for publication.

## Ethical Considerations

The study was approved by the institutional ethical committee.

## Funding support

The author declares that they have no funding support for this study.

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#### How to cite this article

Yaseen, A. H. and Yaaqoob, L. A. (2023). Biosynthesis of cobalt oxide nanoparticles using blood serum as reducing and stabilizing agent for therapeutic use against multi-drug resistant *Acinetobacter baumannii*. *Microbial Science Archives*, Vol. 3(1), 24-31. <https://doi.org/10.47587/MSA.2023.3105>

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