Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i3.5079

MORPHOLOGICAL EFFECTS ON IN-VITRO EVALUATION OF ANTIBACTERIAL ACTIVITY OF MONODISPERSED FINE PARTICLES OF COBALT OXIDE AGAINST VARIOUS BACTERIAL STRAINS

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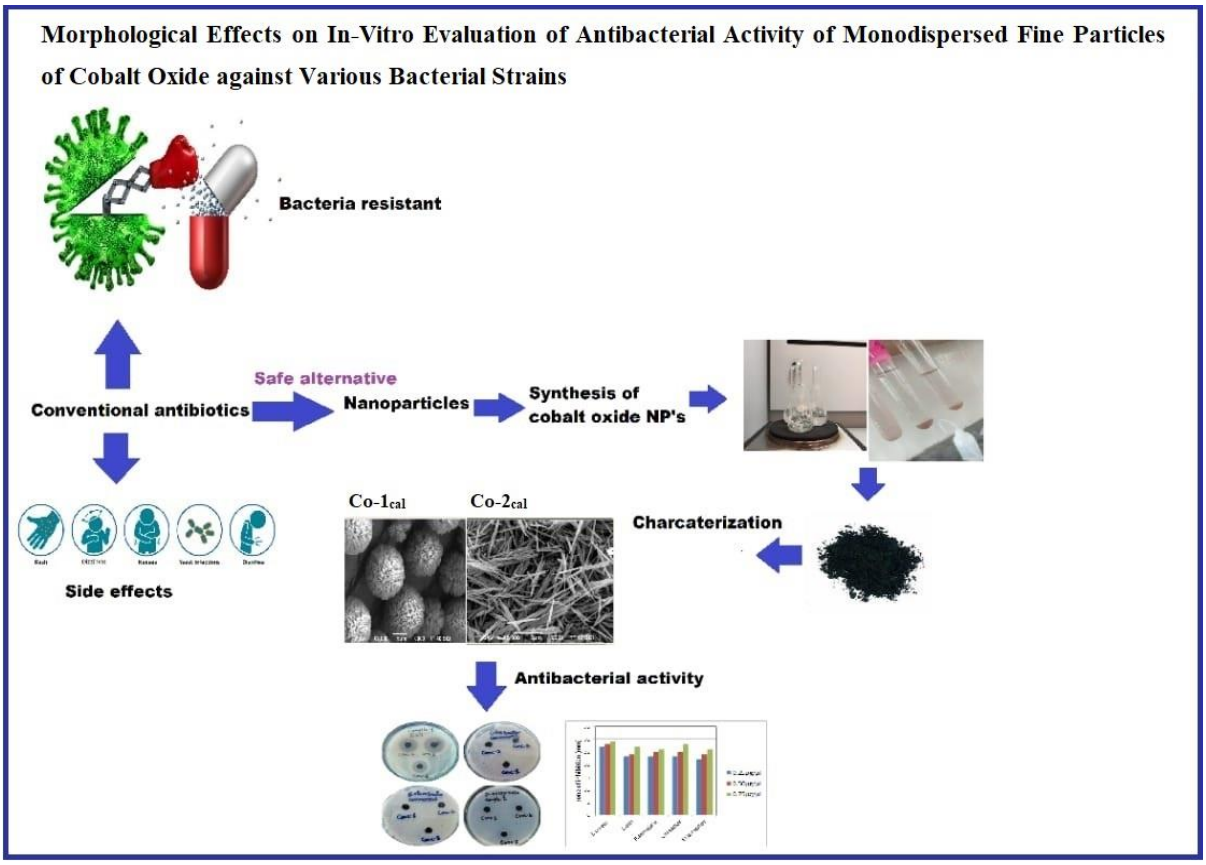
Abstract
Microspheres and nanorods shaped tricobalt tetraoxide (Co_3O_4) fine particles were synthesized using a controlled precipitation method. The precipitation of cobalt acetate and oxalic acid were carried out in ultra-sonic water bath for various duration of time i.e., 20-90°C which caused the formation of cobalt oxalate precipitates. The precursor of Co_3O_4 was then calcined in furnace at 400°C which resulted in the formation of fine particles of Co_3O_4. The effect of reaction time and temperature on particle morphology was studied. The synthesized product was then employed to XRD, SEM and FT-IR analysis. All the employed characterization techniques confirmed the composition, purity, morphology and crystalline nature of the synthesized product. Furthermore, the samples were evaluated for their antibacterial activity using well diffusion method. For this purpose, the antibacterial activity of two selected samples of Co_3O_4 and a commercial Co_3O_4 were evaluated and compared with positive control (ciprofloxacin). The antibacterial study revealed that synthesized Co_3O_4 fine particles possess the potential of an excellent alternative for conventional antibiotics.

Keywords: Cobalt oxide, morphological effects, monodispersed, antibacterial activity, zone of inhibition.



Graphic Abstract

1. Introduction

Nanoscience and nanotechnology is a broad spectrum study of science and technology that mostly deals with the manipulation and control of materials at the nanoscale as well as understanding their morphology and size dependent properties by comparing it with their bulk counterpart.[1]

Nanomaterial forms one of the most important classes of advanced materials. It is a broad area of research and technology that has gained significant importance in the past few decades and its development activity has growing drastically over the last few years.[2] It has revolutionized how these materials are synthesized which enhanced their functionalities that can be accessed in more easy and useful ways. These advanced materials have gained a huge commercial impact in the last few years and will surely increase in the upcoming years.

Nanotubes, nanocomposites, nanowires, and quantum dots are some common terms we most often came across while dealing with nanomaterials. These materials have gained significant advancement due to their enhanced chemical and physical properties over their bulk counterparts due to their varying physiochemical properties such as melting point, wettability, electrical, thermal, and catalytic, these materials show enhanced performance as compared to their bulk. [3, 4]

Currently these materials have found commercial roles in various fields such as scratch-free paints, surface coatings, sports equipment, sensors and in environmental devices. These materials in comparison with their bulk counterparts have greater ratio of particles such as atoms, ions or molecules at nano scale lie on surface which further increases their activity. Nanomaterials have large surface to volume ratio.[5] These properties lead to more sophisticated and valuable product manufacturing. Such as some materials which are diamagnetic in their bulk state shows excellent magnetic behavior when converted into nanoscale. Moreover, materials at nanoscale exhibit excellent thermal and electrical activities i.e., graphene obtained from graphite shows enhanced electrical and thermal properties. Mechanical properties of these NMs are also quite effective as compared to their bulk entities.[6-8]

2. Experimental

2.1 Materials

Cobalt (II) acetate, oxalic acid, ethylene glycol, ethanol, ammonia, acetone, toluene, hydrochloric acid, and nitric acid were obtained from reputed firms. Pyrex glass vessels were used in the course of experimental work for making stock solutions and carrying out reactions. The membrane filter paper was used for filtration purposes. Double distilled water was employed for preparing all types of solutions and for performing reactions.

2.2 Synthesis of precursors of cobalt oxide particles

The precursors of fine cobalt oxide nanoparticles with monodispersed distribution were synthesized through controlled precipitation method. In this process, aqueous solutions of cobalt acetate were mixed in definite ratios with an aqueous solution of oxalic acid at room temperature to form a homogeneous mixture. Ethylene glycol was also added to the mixture of cobalt acetate and oxalic acid in rare cases. These mixtures were then placed in the sonication bath for various duration of time at 20-90°C. All these experiments resulted in the formation of pink colored precipitates in the mother liquor. After precipitation, the reaction mixtures were cooled down to room temperature. The mother liquor was disposed off and the precipitated solid was isolated from the dispersion through membrane filter paper by using vacuum filtration. The collected precipitates were thoroughly rinsed with deionized water and then kept in a desiccator for further study.

2.3 Characterization of Co_3O_4 particles

Morphological evaluation of all the synthesized solids was carried out by SEM (Scanning Electron Microscope SEM: JEOL, JSM-6490). For crystallinity and phase identification, the samples were subjected to x-ray diffractometry (XRD, JOEL JDX-3532). The FT-IR spectroscopic analysis was carried out by using (Schimadzue: IR Prestige - 21, and FT-IR 8400 S) to determine the composition and and functional groups of the synthesized materials.

2.4 Antibacterial activity of Co_3O_4 particles

The antibacterial activity of selected cobalt oxide samples (Co-1 and Co-2) was evaluated against clinical strains of Gram-positive (*Staphylococcus aureus)* and Gram-negative bacterial species (*Pseudomonas aeruginosa, E. coli, Enterobacter cloacae and citrobactor)* by using agar well diffusion method. Fresh cultures of the bacterial strains were prepared at the concentration of 7.5×10^6CFU/50µL and was spread over agar plates having diameter of 8mm. Then the desired particle suspensions at three different concentrations 0.25µg/µL, 0.50µg/µL and 0.75µg/µL were introduced into wells and their antibacterial activity was evaluated and compared with positive control, ciprofloxacin by measuring their zone of inhibitions which was found to increase with increasing concentration of the samples of Co_3O_4.

3. Result and discussion

3.1 SEM analysis of cobalt oxide nanoparticles

3.1.1 Synthesis of cobalt oxide nanoparticles precursors (cobalt oxalate):

The cobalt oxide nanoparticles precursor with confined size were manufactured by the sonochemical process. Following this process, a homogeneous solution was made by mixing aqueous solution of oxalic acid with aqueous solution of cobalt acetate in appropriate amount at room temperature. In some cases, ethylene glycol was also added to the solution of cobalt acetate and oxalic acid. The obtained homogeneous solution was then sonicated for different time periods at 20-90°C. As a result of reaction, the formation of precipitation in the mother liquor, which were separated by vacuum filtration, washed with distilled water and ethanol, dried in air, kept for further characterization. The present study discussed the morphological changes in nanoparticles of cobalt oxalate, obtained at different sonication time. In the given sonochemical process, the concentration of reactants, reaction time, reaction temperature were the key factors that control the cobalt oxalate

morphology. The nature of growth is also important factors that affect the morphology of synthesized nanoparticles. The above discussed parameter which directly control the growth of crystal. There upon, a hard control on these growth parameters may lead to control morphological manufacturing of nanomaterial. When determining the morphology of a product, the nature of crystal formation is a crucial factor. Extensive research on this topic has revealed certain important variables, such as the reaction mixture's composition, temperature, nature of solvent and ageing time. These circumstances were thought to be primarily responsible for controlling the crystal's shape. Therefore, it is thought that by simply adjusting the previously mentioned growth parameters, the structural and morphological properties of the manufactured powders might be tailored. So, if we strictly manage these parameters, we might be able to synthesize nanomaterial under morphological control.

Experiment was performed in order to evaluate the effect of reaction time on the cobalt oxalate nanoparticle morphology, by keeping other parameters constant, only the reaction time was varied. In an experimental setup, 0.4 molar aqueous solution of cobalt acetate and 0.1M aqueous solution of oxalic acid were mixed in appropriate ratio of about 3:7 at ordinary temperature. The obtained homogeneous solution was sonicated at 30°C for one minute and 60 minute, respectively. The resulting precipitates formed at the bottom of the solution were separated by vacuum filtration and characterized. As a result, pink color cobalt oxalate powders were obtained.

The SEM analysis of the calcined powders showed the uniform 3D microsphere morphology with an average diameter of 15(µm). These 3D microspheres were closely packed and retained their morphology up to one minute of sonication. When the sonication time was increased up to certain limit, these morphologies lost their identity and became disintegrated.

Moreover, when the sonication time were exceeded up to 60 minutes, the disintegrated morphology was converted into nanorods. This indicated that the conversion of 3D microspheres to the nanorods was related to the ultrasonic treatment time.

3.1.2 Calcination of cobalt oxalate precursor to cobalt oxide (Co_3O_4) nanoparticles

Calcination of the precursor powders is a common and basic route for the synthesis of metal oxide nanoparticles. The prepared cobalt oxalate nanostructures served as useful precursor for synthesis of cobalt oxide nanoparticles. Both cobalt oxalate samples prepared at 30°C for one minute and 30 minutes' reaction time were converted into the cobalt oxide nanoparticles via heating at high temperature of 400°C in a programmed furnace for 2 hours at the heating rate of 5°C/min. Due to thermal treatment the cobalt oxalate powders were successfully converted into cobalt oxide nanoparticles without any effect on the particle morphology of the synthesized product. These observations showed that the microsphere validate their morphology up to certain limit of ultrasonic irradiation; however, when increase in sonication time caused the disintegration of the microsphere to nanorods. Both the calcined samples were named as Co-1_{cal} and Co-2_{cal} and subjected to further characterization and investigation of antibacterial activity.

3.1.3 SEM Analysis of Co_3O_4

The scanning electron microscopy (SEM) was used to examine the morphology of the cobalt oxide powders obtained after calcination of the precursor at high temperature of 400°C.

Figure 1a shows the microsphere morphology of the sample Co-$_{1cal}$, which is further composed of a number of nanorods cobalt oxide nanoparticles. Furthermore, the SEM micrographs indicated that the microspheres of cobalt oxide nanoparticles were highly closed packed with a size of about 15 µm. Figure 1b shows the nanorods morphology of the cobalt oxide nanoparticles, Co-2_{cal}, which were also obtained by the calcination of the precursor. The obtained SEM micrographs shows that the nanorods of cobalt oxide nanoparticles are uniform in shape and size and exhibit a width of approximately 50-100 nm with a length of 2-3 µm.

3.2 XRD analysis of Co_3O_4

The composition, purity and crystallinity of the calcined samples of Co_3O_4 (Co-1 & Co-2) were evaluated by carrying out X-ray diffractometry. The XRD patterns of both the sample were recorded over 2θ range of 15° to 80°. The presence of sharp diffraction peaks shows crystalline nature of the synthesized product as can be noticed from Figure 2. All the diffraction peaks were in complete agreement with pure cubic structure of Co_3O_4 with JCPDS No. 43-1003 and characteristics reflection lines identified as (111), (220), (311), (222), (400), (422), (511) and (440) respectively. The crystalline size was estimated from major peaks of the obtained XRD- patterns.

The presence of similar diffraction peaks for both samples of Co_3O_4 with different intensities were due to different crystalline arrangements in both morphologies. Absence of other peaks confirmed that the synthesized product is pure tricobalt tetra oxide (Co_3O_4). The crystallite size for both the samples were determined using Debye-Schrerer formula,

$D = k\lambda / (\beta \cos\theta)$

Where, D is crystallite size of particles, K is scherrer constant (0.9), ʎ is wavelength of x-rays used (0.154 nm) β is full width at half maximum of major diffraction peaks.

The average crystallite sizes were estimated to be 15.025 nm and 13.135 nm, respectively.

Which were attributed to variation in degree of crystallinity of both the samples.

3.3 FT-IR analysis of Co_3O_4

To investigate the composition and purity of the synthesized product, both the samples i.e., Co-1_{cal} and Co-2_{cal} were also subjected to FT-IR spectroscopy. The FT-IR spectra obtained in the wavenumber range of 400–4000 cm^{-1} are consisted of a number of absorption bands that occurred as a result of different chemical groups, present on the surfaces of the analyzed particles Figure 3 shows the absorption bands ranging from 3800–3100 cm^{-1} which are assigned to OH stretching vibrations. As well as the band from 1600-1400 cm^{-1} was due to OH bending vibration. The strong absorption peaks at 667cm^{-1} and 572 cm^{-1}indicated that spinel tricobalt tetra oxide (Co_3O_4) was successfully synthesized. The O-H bending and stretching modes of water are said to be responsible for the small absorption peak at 1631 cm^{-1} and the broad band at 3431 cm^{-1}. Figure 3 shows that the FT-IR spectra of both the calcined samples, indicating identical bonding properties of their precursor chemicals. Consequently, the FT-IR investigation of the calcined powders confirmed the aforementioned XRD results.

3.4 Antibacterial activity

The antibacterial activity of fine powders of synthesized tricobalt tetra oxide particles were evaluated against various strains of gram negative (*E-Coli, Enterobactor cloacae, P. aeruginosa and Citrobactor*) and gram positive bacterial strains (*S. aureus*). Although the extent of antibacterial activity varies with morphology, size and concentration of the respective particles but in the present study the antibacterial activity of cobalt oxide was estimated by varying the morphology in addition to concentration of synthesized nanoparticles against different bacterial strains.

Agar well diffusion method was used to estimate the antibacterial activity by measuring the zone of inhibition (mm) which was formed around the wells in petri dish in which the samples were introduced. The wells with specific diameter (mm) were made with sterile gel puncture. Two synthesized samples (Co-1_{cal}, Co-2_{cal}), commercially available Co_3O_4 and a positive control (Ciprofloxacin) having three different concentrations (0.25µg/µL, 0.50µg/µL, and 0.75µg/µL) were employed for antibacterial activity.

The zone of inhibition respective for each concentration and for a specific bacterial strain can be seen from Figure 4. Moreover, commercial samples do not show any antibacterial activity at the selected concentrations and hence did not form any zone of inhibitions.

The obtained zones of inhibition were measured and given in table 2 which shows that tricobalt tetra oxide (Co_3O_4) is an effective antibacterial agent that reduced the bacterial growth to a greater extent

noticing from the diameter of ZOI (zone of inhibition). It is worth to mention that the antibacterial activity of both the samples was comparable to positive control ciprofloxacin.
Furthermore, bar graph given in Figure 5 shows that the antibacterial activity of the synthesized nanoparticles (Co-1_{cal}, Co-2_{cal}) were more toward gram positive bacterial strains than gram negative. This is because of their structural composition. Gram positive bacterial strain has a single peptidoglycan cell wall thus the chances of particle penetrability were high and thus have high chances to suppress the bacterial growth and thus result in larger zone of inhibition around the wells. Thus *S. aureus* is more susceptible to get destroyed by Co_3O_4 nanoparticle which was confirmed by its zone of inhibition. It was found to be larger with diameter of 29 mm and 28 mm for at concentration of 0.75µg/µL, respectively.
On the other side, gram negative bacterial strains have an extra membrane made of lipopolysaccharide which lower the inhibitory action of these nanoparticles on these bacteria and hence result in smaller zone of inhibition as compared to gram positive bacterial strains, which is shown by diameter of their zone of inhibition which was found to be smaller as compared to *S. Aureus.* Furthermore, based on significant antibacterial activity shown by the synthesized particles of Co_3O_4, the obtained results were then compared with results reported by other researchers working in this field and given in Table 3. The comparative study revealed that the particles synthesized in the present work showed better antibacterial activity at such low concentration 0.25µg/µL of the particles employed and possess the potential to be used as safe alternative for conventional antibiotics.

Thus it is concluded that the antibacterial activity of these NPs along with concentration also depends on the structure, composition and membrane arrangement of the targeted bacteria. Researchers have put forward various mechanisms that explains the actions of these NPs on the bacterial cells such as,
1. Generation of reactive oxygen species (ROS).
2. Metal ions release (Co^{+2})
3. Induced damaged by electrostatic interaction of metal oxide particles on bacterial cell wall.
Each mechanism causes certain degree of bacterial damage and hence leads to bacterial cell death. Although the exact mechanism is still ambiguous to the researcher's community however in the present study it is believed that the bacterial cell death is due to interaction of tricobalt tetra oxide (Co_3O_4) nanoparticles with the bacterial cell wall by electrostatic interaction, this interaction results in penetration of NPs inside the bacterial cell that caused production of reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide (O^{-2}) and hydroxyl radical (OH^-). These species aggregate on the surface of bacteria and result in membrane destruction and penetrate inside the cell destroying the intracellular content of cell including DNA and proteins and hence result in bacterial cell death.

4. Conclusion

- Monodispersed 3D microspheres (Co-1_{cal}) and nanorods shape (Co-2_{cal}) tricobalt tetra oxide (Co_3O_4) fine particles were synthesized using controlled precipitation method.
- The synthesized particles were confirmed by XRD analysis with crystalline nature and crystallite sizes of 15.03 and 13.35 nm for Co-1_{cal} and Co-2_{cal}, respectively.
- The antibacterial activity of the synthesized product was evaluated by agar well diffusion method and was compared with commercial (Co_3O_4) and positive control ciprofloxacin.
- The antibacterial activity of both the samples measured was found to increase with increase in Co_3O_4 particles concentration against various bacterial strains.
- The antibacterial activity of both the samples was comparable to positive control ciprofloxacin while the commercial Co_3O_4 sample showed no inhibition zone and thus no antibacterial activity at the selected concentration range of NPs.

- The measured antibacterial activity of the synthesized nanoparticles ($Co\text{-}1_{cal}$ & $Co\text{-}2_{cal}$) were more toward gram positive bacterial strains than gram negative bacteria.
- Thus the synthesized cobalt oxide NPs might be used as an effective and safe alternative for conventional antibiotics.

Acknowledgement:
The authors would like to acknowledge the Deanship of Graduate studied and scientific Research, Taif University for funding this work

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Figures

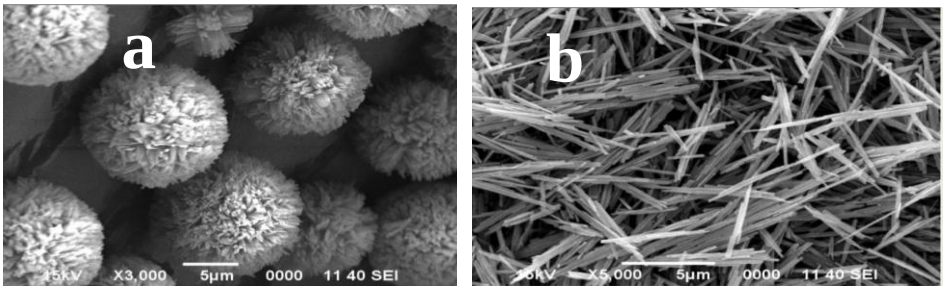

Figure 1. SEM micrograph of cobalt oxide (Co_3O_4) calcined at 400°C (a) Co-1_{cal}: microspheres (b) Co-2_{cal}: nanorods

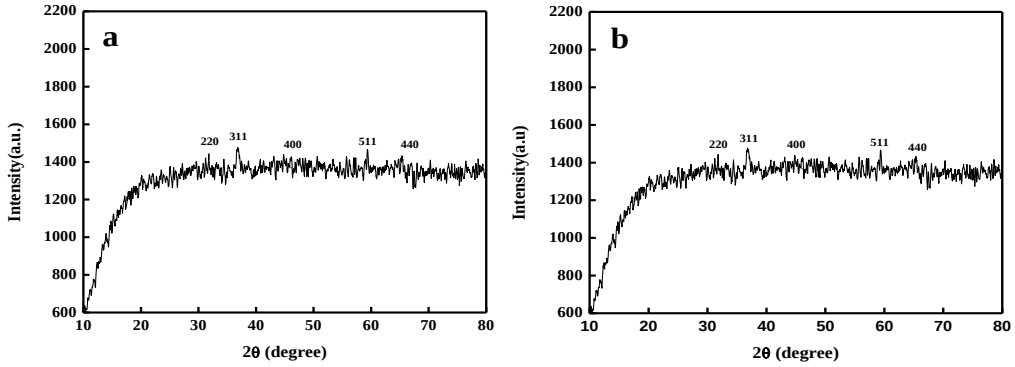

Figure 2. XRD pattern of calcined particles of cobalt oxide (Co_3O_4) (a) Co-$1_{cal.}$ (b) Co-$2_{cal.}$

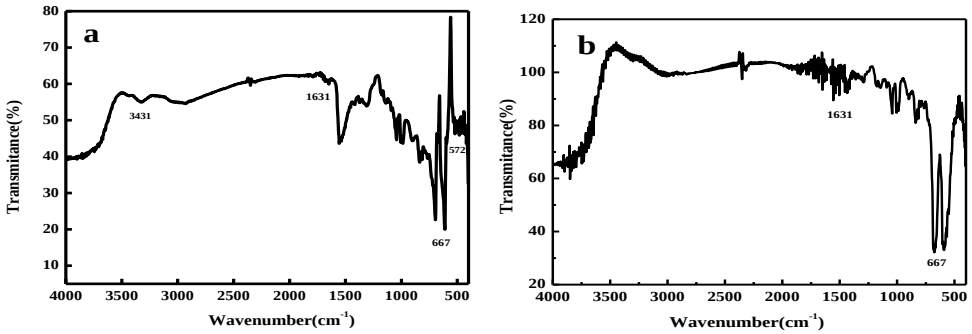

Figure 3. FT-IR spectra of the Co_3O_4 samples (a) Co-1_{cal} (b) Co-$2_{cal.}$

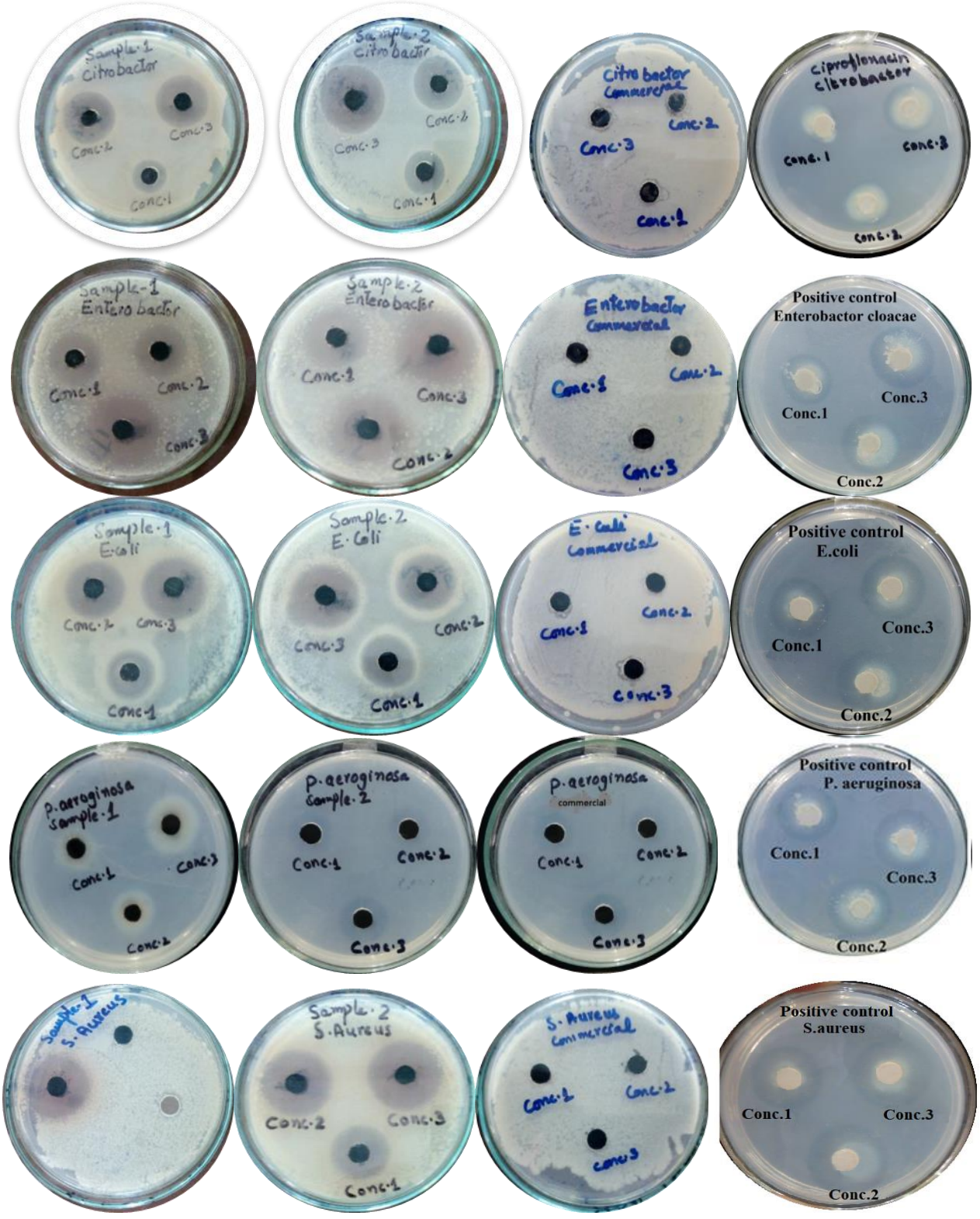

Figure 4. Zone of Inhibition of Co_3O_4 and positive control against various Gram-negative and Gram-positive bacterial strains (Conc-1: 0.25 µg/µL, Conc-2: 0.50 µg/µL, Conc-3: 0.75µg/µL)

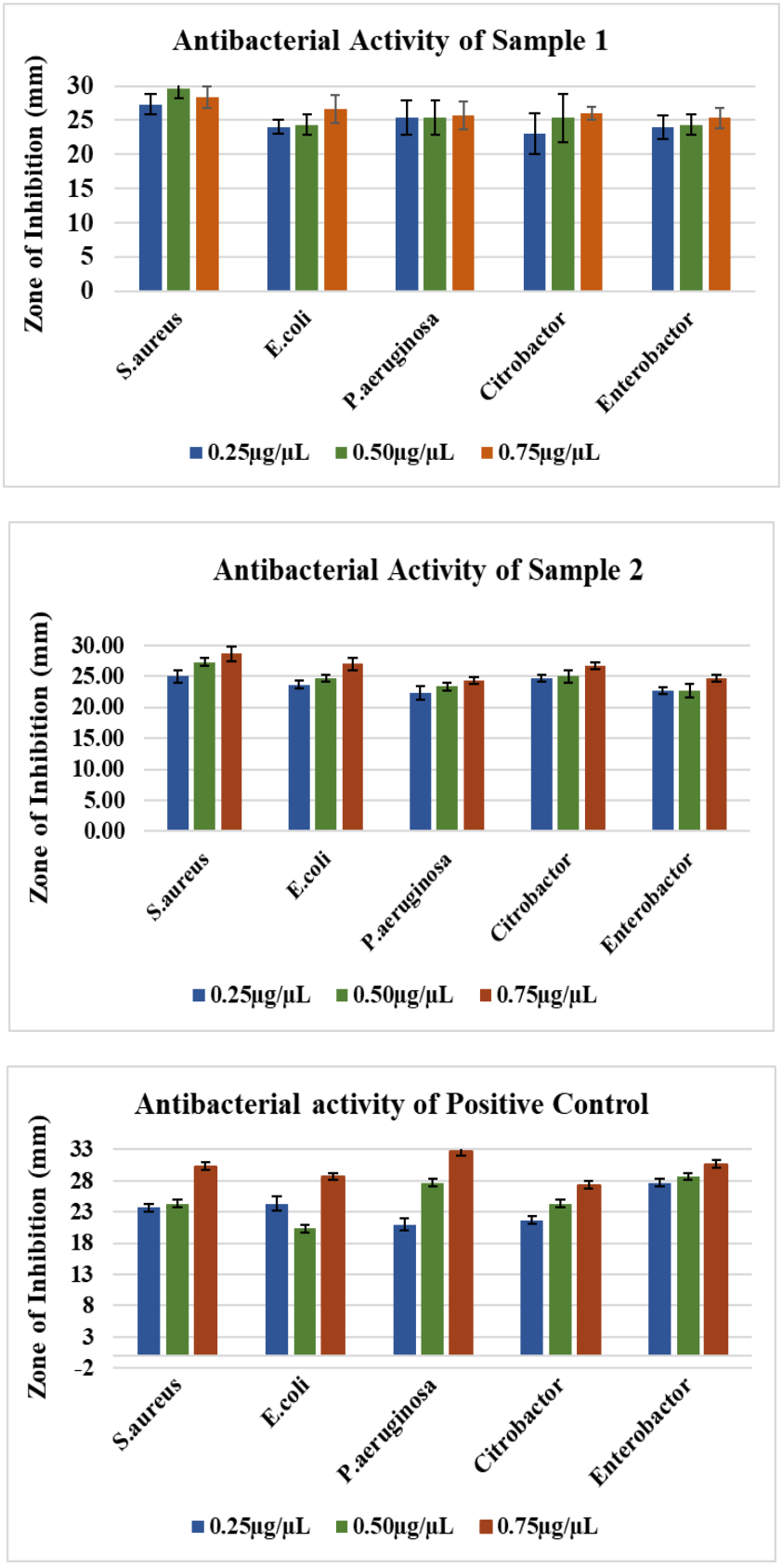

Figure 5. Bar graph showing effect of particle morphology and c oncentration on antibacterial activity of selected Co_3O_4 samples (Sample1: Co-1_{cal}, Sample 2: Co-2_{cal}) and positive control

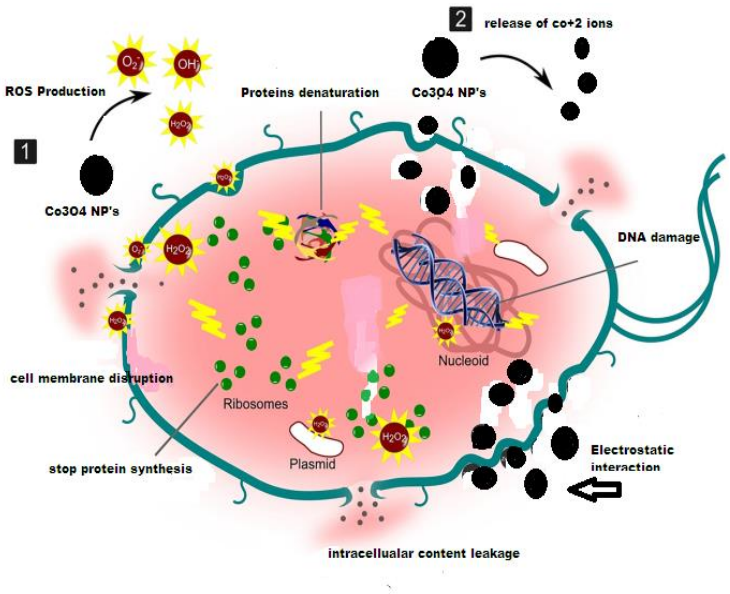

Figure 6. Schematics illustrating proposed mechanism of action of Co_3O_4 NP's

Table 1. Crystallographic parameters of calcined samples Co-1_{cal}, and Co-2_{cal}, calculated from XRD patterns

Sample code	2θ(°)	θ/rad	FWHM(°)	Crystallite size (nm)	Average crystalline size
Co-1_{cal}	31.350	0.3153	0.45	16.98	15.03 ±0.5nm
	36.748	0.2761	0.56	13.35	
	44.79	0.3002	0.45	16.28	
Co-2_{cal}	31.50	0.3165	0.55	13.9	13.35 ±0.2 nm
	36.748	0.2774	0.55	13.67	
	44.79	0.3003	0.58	12.54	

Table 2. Antibacterial activity of selected Co_3O_4 nanoparticles and Positive control

Zone of inhibition(mm)									
Bacterial strains	**Co-1_{cal}**			**Co-2_{cal}**			**Positive control**		
	0.25 µg/µL	**0.50 µg/µL**	**0.75 µg/µL**	**0.25 µg/µL**	**0.50 µg/µL**	**0.75 µg/µL**	**0.25 µg/µL**	**0.50 µg/µL**	**0.75 µg/µL**
S. aureus	27±0.2	28±0.4	29±0.2	26±0.2	28±0.2	30±0.4	23±0.3	24±0.2	31±0.2
E. coli	23±0.3	24±0.5	27±0.2	24±0.1	25±0.3	26±0.3	25±0.1	20±0.2	29±0.1
Enterobactor cloacae	22±0.2	24±0.3	26±0.1	23±0.1	24±0.2	25±0.2	20±0.1	28±0.4	33±0.3
Citrobactor	23±0.2	25±0.4	28±0.5	25±0.2	26±0.2	27±0.1	22±0.3	25±0.1	27±0.5
P. aeruginosa	23±0.1	25±0.1	26±0.2	23±0.2	24±0.4	25±0.3	28±0.2	29±0.1	31±0.2

Table 3. Comparative study of antibacterial activity of selected Co_3O_4 nanoparticles NPs with literature

S. No.	Synthesis Method	Morphology	Test method for antibacterial activity	Bacterial species	ZOI (mm)	Reference
1	Chemical reduction method	Irregular	Agar disk diffusion method	*E-coli, P-aeruginosa, Bacillus subtillis*	12mm 14mm 16mm	[32]
2	Co-precipitation method	Spherical and irregular shaped	Agar well diffusion method	*E-coli, P-aeruginosa,*	16nm 14nm	[34]
3	Thermal treatment	Spherical	Disk diffusion method	*E-coli* *Bacillus subtillis*	22mm 20mm	[35]
4	Thermal decomposition	Spherical and irregular shaped	Agar well diffusion method	*E-coli* *S-aureus*	12mm 13mm (Conc. 16µg/µl)	[31]
5	Calcination method	Spherical	Agar well diffusion method	*S-aureus* *P-aeruginosa,* *E-coli*	12-16mm	[27]
6	Sol-gel method	Spherical	*MIC and MBC method*	*E-coli,* *P-aeruginosa* *S-aureus*	16-18mm	[42]
7	Co-precipitation	Nanotubes	*Disk diffusion*	*E-Coli*	15mm-14.6mm	[14]

	method			*S-aureus*		
8	Urea based thermal decomposition	Irregular	Disk diffusion method	*S-aureus* *P-aeruginosa* *E-coli*	22-26mm	[33]
9	Chemical synthesis	Spherical	Agar well diffusion	*E-coli* *S-aureus*	32mm 33mm	[31]
10	Co-precipitation	Spherical	Disk diffusion method	*S-aureus*, *P-aeruginosa* *E-coli*	7-12mm	[43]
11	Sol gel	Spherical	Agar well diffusion method	*E-coli* *P-aeruginosa* *S-aureus*		[39]
12	Co-precipitation	Microspheres	Agar well diffusion method	*E-coli*, *S-aureus* *P-aeruginosa* *Citrobactor* *Enterobactor cloacae*	23 27 23 23 22 0.25µg/µl	Present work
13	Co-precipitation	Nanorods	Agar well diffusion method	*E-coli* *S-aureus*, *P. aeruginosa* *Citrobactor*, *Enterobactor cloacae*	24 26 23 26 23 (Conc. 0.25µg/µL)	Present work