Synergistic Effects of Cobalt Oxide Combinations With Chitosan for Enhanced Antibacterial Activity

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**Abstract:** Due to the overuse of antibiotics along with the evolution of bacteria resistance, antibiotic resistance is a major contribution to infection related mortality in developing countries. Antibiotic resistance is growing despite the fact that antibiotics have had a huge influence on medicine; this is primarily due to microbiological adaptation. Chitosan, an antibacterial cationic polymer, and cobalt oxide nanoparticles (CoO NPs) are being investigated for their potential in antimicrobial applications in order to alleviate this problem. CoO NPs' distinct physical and chemical characteristics have made them promising in a number of industries, including medicine. These include their antimicrobial and magnetic qualities. Chitosan's usefulness as an antibacterial agent is increased by its capacity to impede bacterial development and by its application in environmental purification.In this study the aim is to determine if Cobalt Oxide combinations with Chitosan are capable of enhancing antibacterial properties. Consequently, the goal of this research is to maximise the production of Cobalt Oxide Nanoparticles for use as an antibacterial agent in combination with chitosan.In this study, chitosan infused cobalt oxide particles were synthesised using the chemical precipitation method to pose an obstacle to antibiotic resistant bacteria. The nanoparticles demonstrated strong antibacterial action against both gram positive and gram negative bacteria.

**Keywords:** Cobalt Oxide, Chitosan, Antibacterial activity, Nanoparticles, Enterococcus, E.coli

# Introduction

One of the main causes of death in developing countries is infection [(Aparna et al., 2021; Poornima et al., 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/gMMe66/q85Gu+Lctl8+ITnnS). This is mostly because of the rise of antibiotic resistance, which is primarily caused by the emergence of novel pathogenic pathogens. Bacteria have become more intelligent as they have changed over time [(Merchant et al., 2022; Pandiyan et al., 2022)](https://paperpile.com/c/gMMe66/ZGlB+nKiN). Bacterial resistance to antimicrobial medications has increased as a result of healthcare facilities' abuse of antibiotic [2022)](https://paperpile.com/c/gMMe66/ZGlB+nKiN+ikn7h). Antibiotic discovery raised hopes for the control and prevention of illnesses. Nonetheless, in impoverished countries, infections continue to be the main cause of death. This is brought on by the advent of novel diseases, the reappearance of illnesses that were previously under control, and most notably, the emergence of antibiotic resistance [(Chokkattu et al., 2022; Ramamurthy et al., 2022)](https://paperpile.com/c/gMMe66/XxvmV+62ngt). Antimicrobial resistance is recognized as a major problem in the treatment of microbiological infections in both hospitals and the general public, and it appears that almost every new drug will eventually cause it to occur[(Kapoor et al., 2017)](https://paperpile.com/c/gMMe66/w40P). Antibiotics were one of the biggest discoveries made by humans in the 20th century[(Jain & Verma, 2022; Marya et al., 2022)](https://paperpile.com/c/gMMe66/DudUh+vTR0a). Antibacterial success transformed modern biomedicine and aims to define, shape, and expand its limits as well as its possibilities [(Rehman et al., 2015)](https://paperpile.com/c/gMMe66/R880). Unfortunately, the potential for resistance to develop in any therapeutic medication restricts its efficacy. Since resistance undermines an antibiotic's effectiveness and therapeutic effect, the next generation of antibiotics needs to be produced [(Livermore, 2003)](https://paperpile.com/c/gMMe66/rnUV). Resistance to an antibacterial medication such as antibiotics is the term used to describe a pathogen's increased resistance to the standard therapy that was previously effective but vulnerable to it [(Wadhwani et al., 2022)](https://paperpile.com/c/gMMe66/7FdCS). Antibiotics have revolutionised medicine and saved many lives since they were first introduced at the turn of the 20th century[(Muteeb et al., 2022)](https://paperpile.com/c/gMMe66/xqDH). However, the concerning rise in antibiotic resistance looms over the advancements in antibiotic discovery [(Sreevarun et al., 2023)](https://paperpile.com/c/gMMe66/4mUWw). This worldwide calamity is the result of continuous microbial adaptability brought on by the misuse and overuse of antibiotics[(A. U. Khan & Rehman, 2016)](https://paperpile.com/c/gMMe66/AZfN). Every living thing requires cobalt for proper metabolism because it is an ultra-trace metal that is mostly found in cobalamin, generally referred to as vitamin B12 . For many years, metal oxide nanoparticles have been the focus of intense research in a variety of disciplines, including medicine [(Subramanian & Harikrishnan, 2023)](https://paperpile.com/c/gMMe66/hmYuh). Due to the unique physical and chemical characteristics of cobalt oxide nanoparticles, which are based on the growing physiological functions of cobalt, these particles have been used in a variety of sectors[(Huang et al., 2021)](https://paperpile.com/c/gMMe66/JsL4) . In a comparable manner, cobalt oxide nanoparticles appear to have potential for a wide range of biomedical uses and may likewise have significant effects on human well-being. Nanotechnology has steadily but slowly taken over a number of sectors worldwide[(Moradpoor et al., 2019)](https://paperpile.com/c/gMMe66/qhfz). This rapid pace of technical advancement is especially noticeable in industrialised countries, where nanotechnology markets have grown in the previous ten years [(Abbasi et al., 2021)](https://paperpile.com/c/gMMe66/ducz). Given how quickly nanoscience is evolving, nanoscale production will soon be included into almost all academic disciplines and technological advancements [(S. Khan et al., 2015)](https://paperpile.com/c/gMMe66/Ny2C). Over time, successful applications of nanotechnology in healthcare have raised people's quality of life. Because of this, a whole new discipline called nanomedicine has developed, enabling researchers to create better ways to prevent, screen for, treat, diagnose, and take preventative healthcare actions[(Malik et al., 2023)](https://paperpile.com/c/gMMe66/a9Ux). Chitin is alkaline N-deacetylated to produce chitosan, a cationic polymer [(Solanki et al., 2023)](https://paperpile.com/c/gMMe66/HKHzL)

Proteins are recovered from waste products from food processing and heavy metals are chelated from wastewater by using chitosan's cationic characteristic[(Gerhard Vogel et al., 2010)](https://paperpile.com/c/gMMe66/uzBQ). Due to their ability to efficiently inhibit the development and replication of dangerous bacteria as well as the spread of toxic pollutants, chitosan and its derived compounds are known as functional materials for environmental purification[(Vermeulen & De Smet, 2012)](https://paperpile.com/c/gMMe66/cwop). One of chitosan's most studied properties is its antibacterial activity, which finds applications in food, medicine, and cosmetics [(Ganapathy 2021)](https://paperpile.com/c/gMMe66/AtEbZ). Through both in vivo and in vitro communication with chitosan in various forms, chitosan has been studied as an antibacterial substance towards an extensive variety of target microbes such as algae, bacteria, yeasts, and fungi [(Chokkattu et al., 2023)](https://paperpile.com/c/gMMe66/kOUU7). The antibacterial activity of chitosan is known to be affected by several variables that work in a systematic and independent manner. The most well-known way that chitosan has an antibacterial impact is by attaching itself to a negatively charged cell wall, which disrupts the membrane and alters its permeability. The adhesion to DNA that follows prevents DNA replication and ultimately leads to cell death [(Laghari et al., 2023; Ramakrishnan et al., 2023)](https://paperpile.com/c/gMMe66/gf7Pb+hplqP). Another tenable theory is that chitosan acts as a chelating agent, attaching itself to trace metals to create poisons and prevent the growth of microorganisms. Among existing transition metal oxides, Cobalt Oxide can be defined as a black powdered substance with magnetic as well as antibacterial traits. The particles with magnetic properties that are independent and have a maximum diameter of 100 nm are known as magnetic nanoparticles [(Muthuswamy Pandian et al., 2022; Ramakrishnan et al., 2023)](https://paperpile.com/c/gMMe66/8Y9cy+gf7Pb). Cobalt is a semistable metal with three distinct crystalline phases that make it one of most strongly significant magnetic materials. Phases such as the epsilon phase, face-centred cubic (FCC) phase, and hexagonal closed packed (HCP) phase are the three crystalline cobalt oxide phases . Based on the traits, Cobalt Oxide Nanoparticles are applied in a large variety of fields, consisting of biochemistry, sensors, magnetic materials, electrolytic systems, smart absorbers, and medicine . Given that cobalt oxide nanoparticles are used in many different fields, optimizing their manufacture seems essential for their possible uses. Through regulating the key factors during its process of synthesis, nanoparticles' sizes, shapes, surfaces, and morphologies can all be enhanced[(Ahmed et al., 2008)](https://paperpile.com/c/gMMe66/YP1H). Cobalt Oxide Nanoparticles have garnered large interest because of many features, including its antifungal, photochemical, high catalytic, and antibacterial activities. Plants, fungi, bacteria, and algae have all been used in the development of green nanoparticle manufacturing techniques. In this field, cobalt oxide biomedical applications are used on a regular basis in a variety of processes, including gene transfer, biosensors, bioimaging, and medicine administration. Furthermore, because of their toxicity and appearance, cobalt oxide nanoparticles can be used as smart weapons against a variety of drug-resistant microorganisms and are a skilled antibiotic substitute. Recent findings have indicated that chitosan possesses features that expedite wound healing, hemostasis, and scar protection, making it a desirable therapeutic biomaterial. The hydroxyl and amino functional groups included in chitosan's molecular structure operate as effective templates for connections to form, enabling the connected structures present in chitosan-based nanocomposites. As a result, chitosan and metal oxides can react chemically to form nanocomposite complexes. Thus, tailoring and modifying the characteristics of nanoparticles in accordance with their role and activity can be taken into consideration. Consequently, the goal of this research is to maximise the production of Cobalt Oxide Nanoparticles for use as an antibacterial agent in combination with chitosan [(Nagy et al., 2011)](https://paperpile.com/c/gMMe66/ng0G)

# Materials and Methods

## Materials

The following are the main components of the materials used in this study: Cobalt Oxide and Chitosan. To prepare Cobalt Oxide, elements such as Cobalt Nitrate, Ethanol, Sodium Hydroxide, Double distilled water and Acetone. To further derive the nanocomposite complex, Chitosan, Acetic acid, and Sodium Hydroxide pellets were used. The apparatus utilised for this research includes, electronic balance, beaker, burette, ice bath, stirrer, centrifuge, hot air oven, and litmus pH paper.

## Method

Finding out if cobalt oxide and chitosan combinations can improve antibacterial qualities is the goal of this investigation. This study was conducted using a technique called the chemical precipitation method. Among the several methods for producing nanoparticles, chemical precipitation is one of the most promising methods for producing nanocatalysts since it allows metal ions to fully precipitate. It is also a popular method for producing nanoparticles with a larger surface area for improved antibacterial activity [(22)](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5931324/).

### Preparation of Cobalt Oxide Nanoparticles

Two beakers were needed to prepare the cobalt oxide nanoparticles. 30ml of boiling ethanol was used to dissolve 0.5g of cobalt nitrate that had been weighed for the first beaker. An ice bath was then used to chill this down to 4oC. After that, 10 mmol of sodium hydroxide was dissolved in ethanol in a second beaker. After then, this solution was moved to a burette. After that, drops of the prepared solution were added to the cobalt solution until it was fully dissolved. During this a white precipitation can be observed which starts to form a milky suspension. This beaker was then kept on the stirrer for a duration of 30 minutes. This aids in the formation of complete milky suspension. Once this was achieved, it was further kept in the stirrer for another 24 hours to allow a heavy precipitation formation. Once this is complete, the precipitate is then kept in the centrifuge to undergo separation. The sample is first washed with double distilled water in the first round, followed by ethanol and then acetone; each round lasting for 10 minutes. This process causes the powder to settle at the bottom. The water is then separated from the powdered precipitate so that the sample can be put in the hot air oven at 80oC for drying. After that, a procedure known as calcination is employed. Applying heat to a solid chemical material (such as mixed carbonate ores) in order to eliminate impurities or chemical compounds or induce thermal breakdown is known as calcination. With a small amount of ambient oxygen present, the compound is heated to greater temperatures without melting. The sample is calcined for two hours at 500oC as part of this procedure.

### Preparation of chitosan colloid

For the preparation of chitosan colloid, 0.1g of chitosan was weighed and dissolved in 100ml of acetic acid. On immediate addition of chitosan a crystal appearance is observed however the chitosan should be mixed until a completely dissolved pale solution of chitosan colloid is obtained.

### Preparation of nanocomposite complex

To the prepared chitosan colloid, 0.1g of previously prepared cobalt oxide was added. The solution is then put on the stirrer for 30 minutes. After it was finished, litmus pH paper was used to measure the solution's pH level. Due to the addition of 100ml of acetic acid during preparation of chitosan colloid the current pH of the solution should be around 1-2 pH turning the pH paper dark pink. However the pH level required is an alkaline pH of 10. To achieve this, sodium hydroxide pellets of concentration 1mol were added to the prepared solution to bring the pH level to 10. When testing with pH paper a dark green colour will be observed. Following this the solution is put in the centrifuge. The solution is first washed with double distilled water in the first round, followed by ethanol and then acetone; each round lasting for 10 minutes. Once dried at 50oC for 1 hour the prepared sample forms a nanocomposite complex of cobalt oxide chitosan nanoparticles.

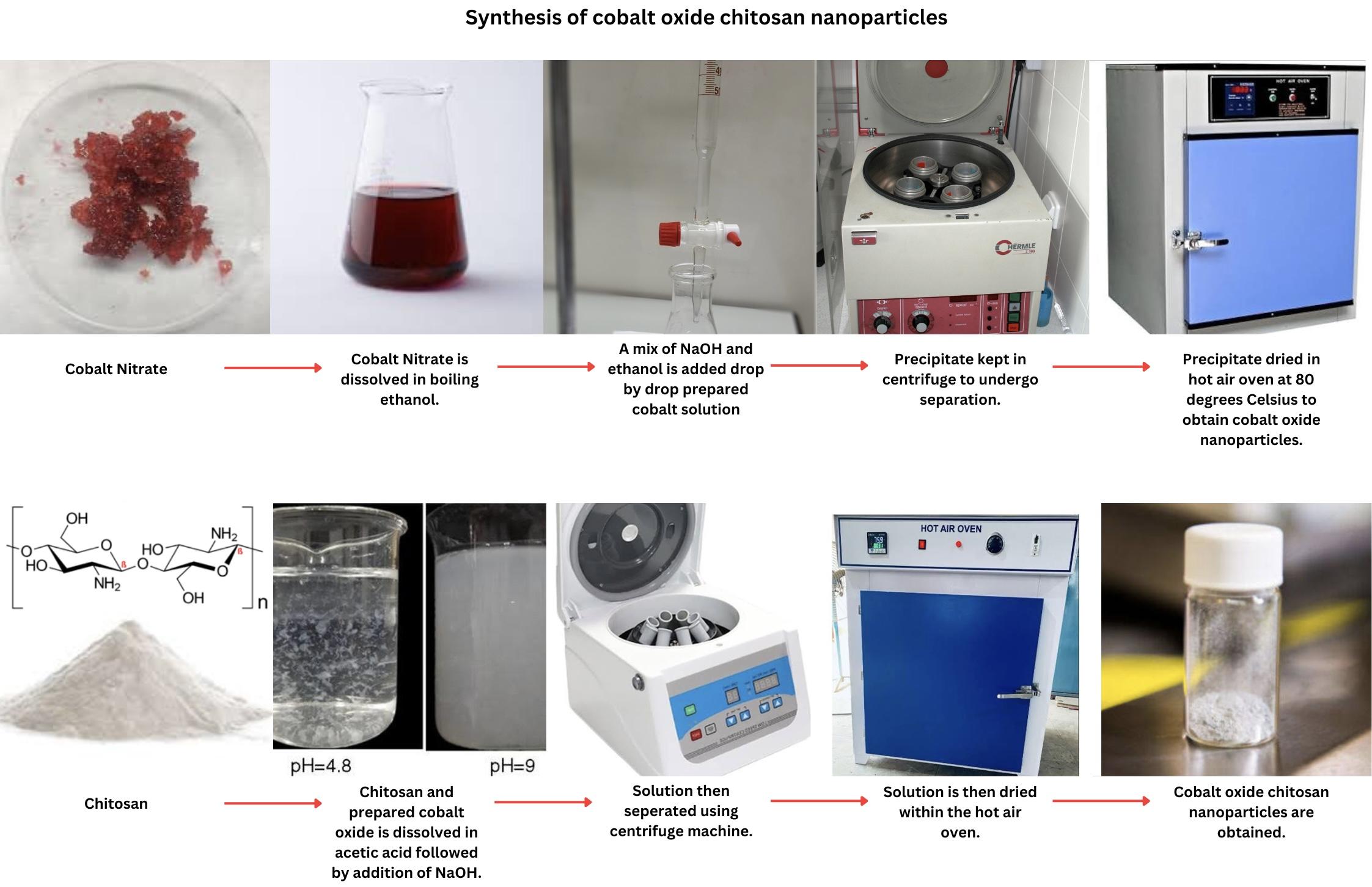


Fig.1 - Method for preparation of Cobalt oxide chitosan nanoparticles

### Characterization

A number of analytical tests must be performed for characterization in the hopes of deciphering the research's mechanism(Chehelgerdi et al., 2023). First of all, an XRD; X-ray crystallography is a technique used to determine the orientation and configuration of atoms within a crystallised substance. To do this, x-ray beams are sent through a crystal, causing the light to diffract in various directions. The next step is that polymeric, organic, and inorganic materials can be recognized using Fourier-transform infrared spectroscopy (FTIR). This technique analyses samples using infrared light to look for certain chemical characteristics.Similar methods include transmission electron microscopy (TEM testing), which creates a picture for additional study by passing an electron beam through a material.

# Results and Discussion

## XRD

XRD was used to determine the crystal structure for produced CS-CoO nanoparticles. In **Fig.1**, the diffractogram is displayed (Saadh et al., 2024). Characteristic peaks for CoO NPs were shown for bare CoO and CS-CoO nanoparticles at 2h = 22.080°, 36.490°, 43.200°, 52.600°, 70.190°, and 77.500°. These peaks are indicated from respective indices (111), (220), (311), (400), (511), and (440) in accordance with the JCPDS card no. 73-1701. The produced NPs contained CoO crystals, as shown by the XRD data.

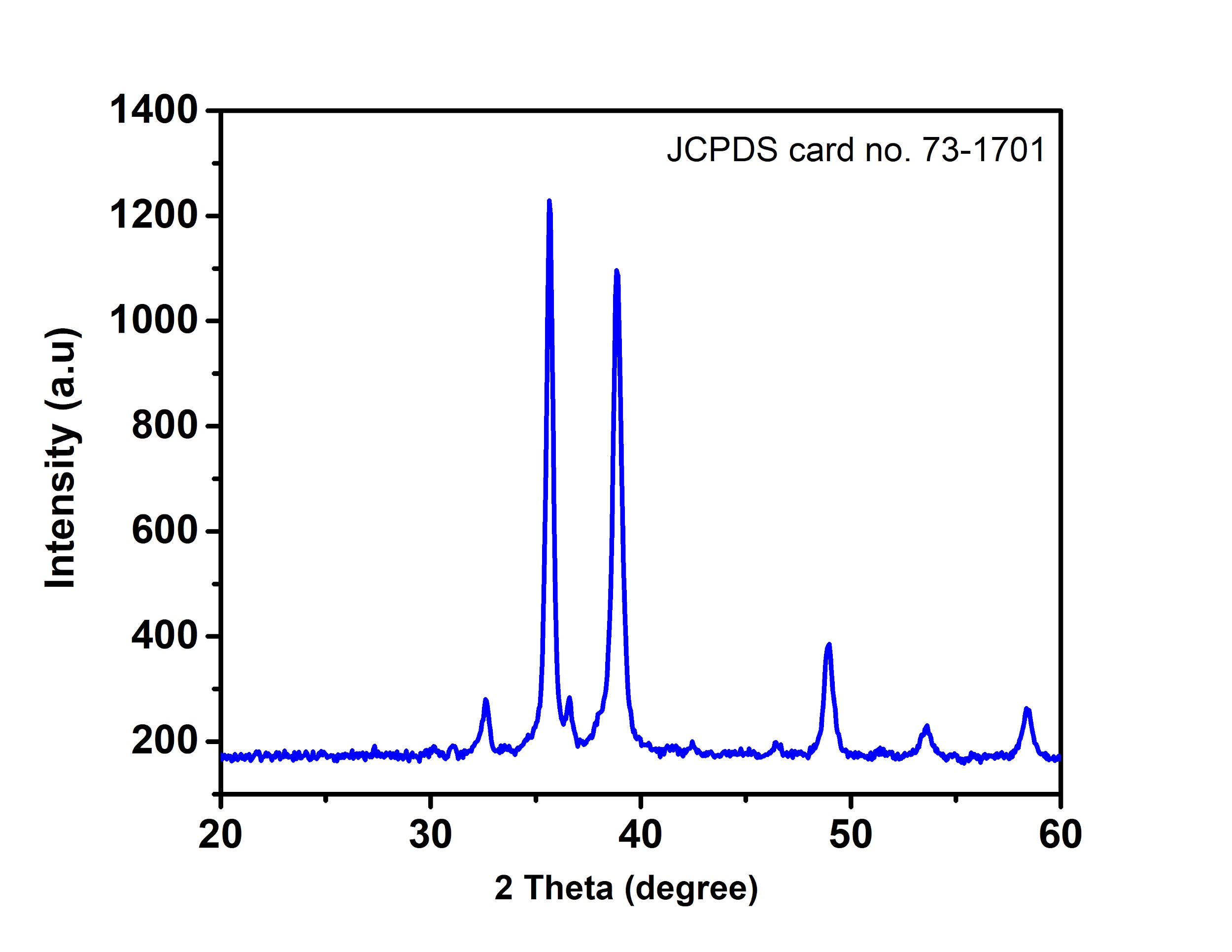


Fig.2 - XRD Graphical analysis

## FTIR

By using FTIR spectroscopy, the association of chitosan with cobalt oxide NPs were examined. Figure 2 displays the FTIR spectrum of the NPs, chitosan, and pure CoO. The presence of the Co–O vibration is indicated by the distinctive infrared band of CoO at 506 cm-1 seen in the FTIR spectra, along with a broad band surrounding it. The spectra of chitosan and the NPs show the presence of peaks at 1,615 ± 15 cm-1, which are associated with NH2 group scissoring. This indicates that the chitosan polymer coating of CoO NPs on the NP surface was accomplished successfully. The band at 1,628 cm-1 in the spectra of chitosan (Fig. 2) is attributed to NH2 group scissoring, while the peak in 1,156 cm-1 is attributed to C–N stretching. The NPs spectra (**Fig. 2**) shows peaks at 1,435 and 1,612 cm-1. The outcomes showed that the chitosan coating of CoO NPs was successful.

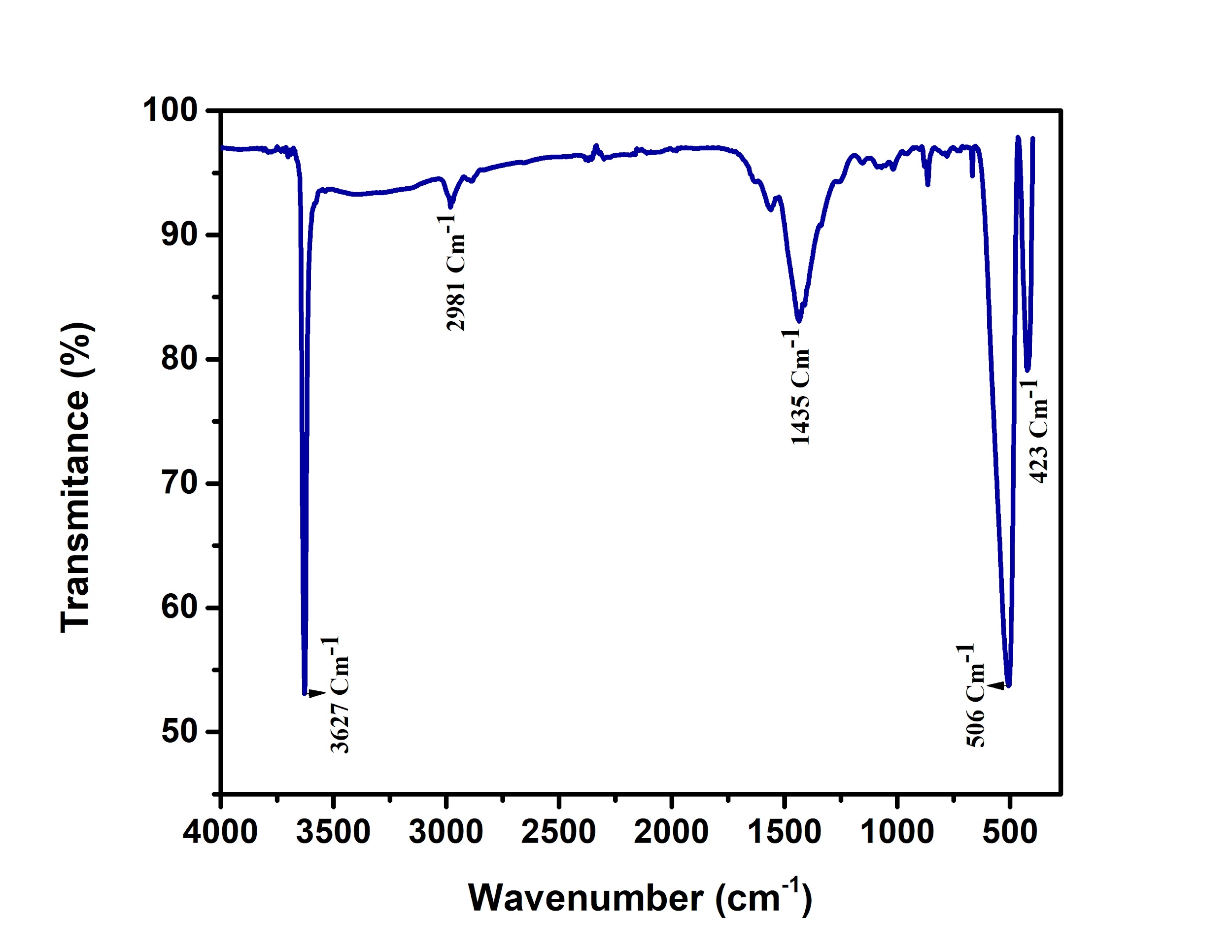
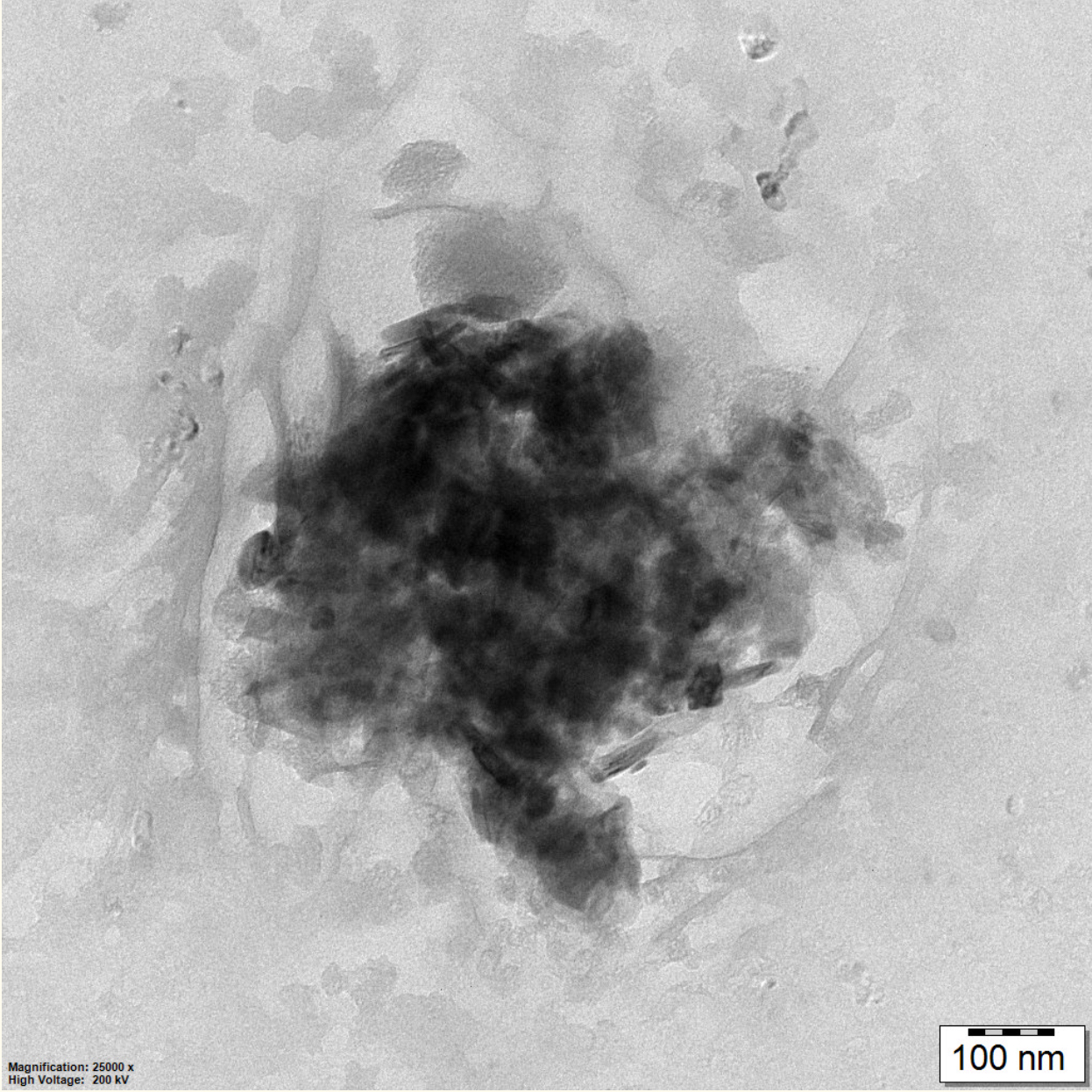
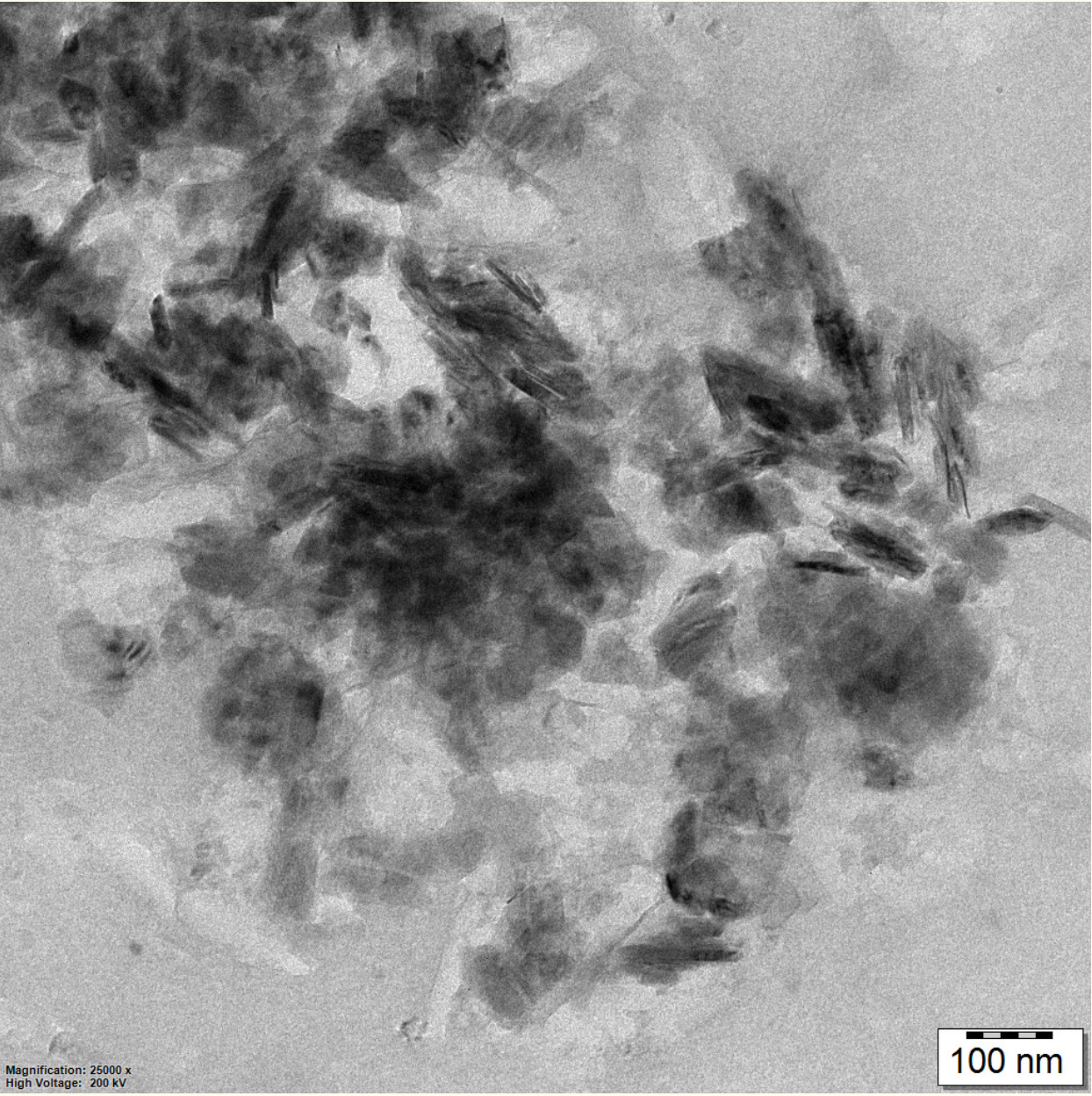
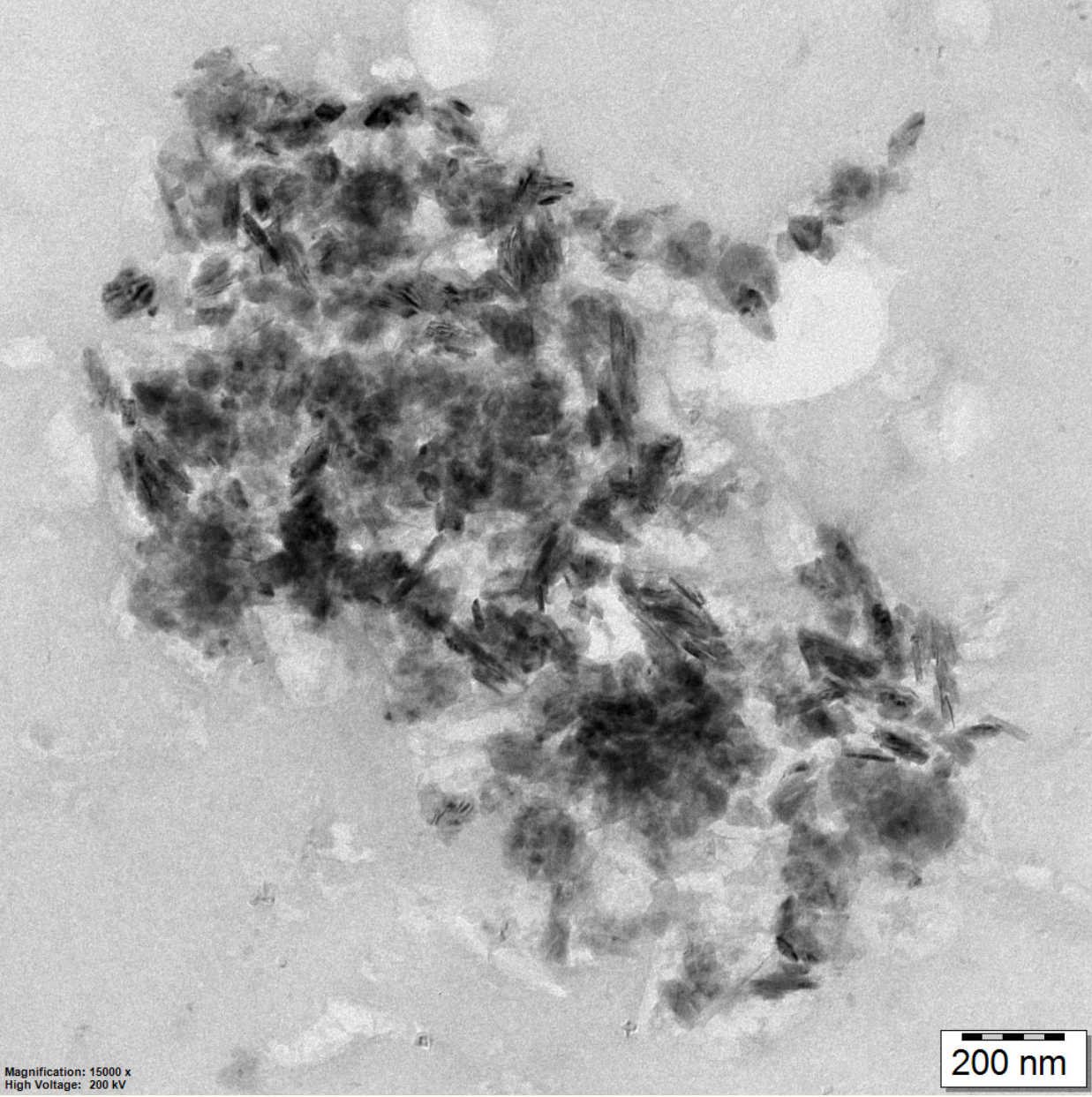


Fig.3 - FTIR Graphical analysis

## HRTEM

According to high resolution transmission electron microscopy, CS-CoO NPs have a mean size of 62 ± 6 nm and are shaped like stacked rods. The outcome is seen in **Fig.3**. The sample's size in its dry state was determined using high resolution transmission electron microscopy.



1. (b) (c)

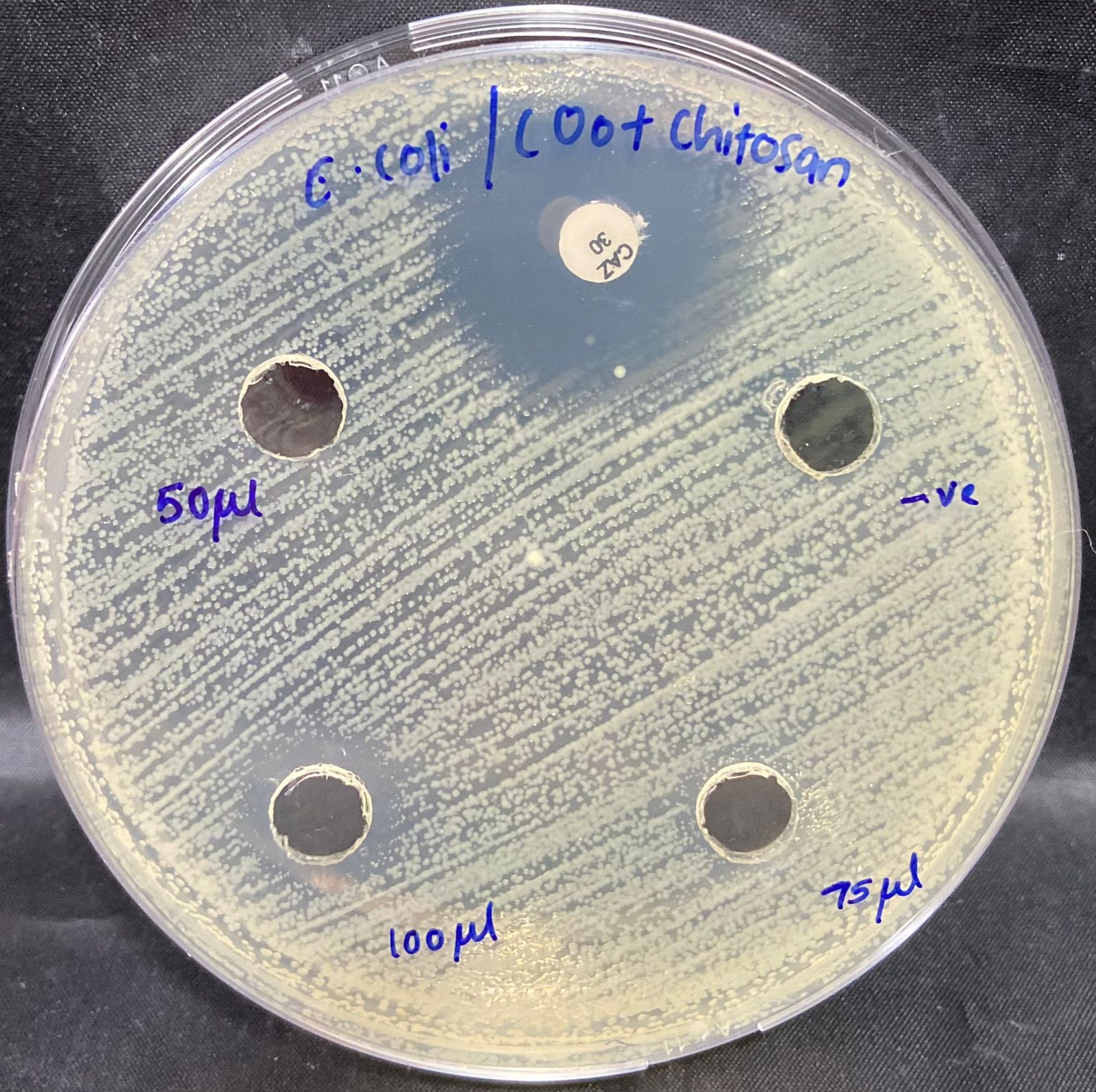
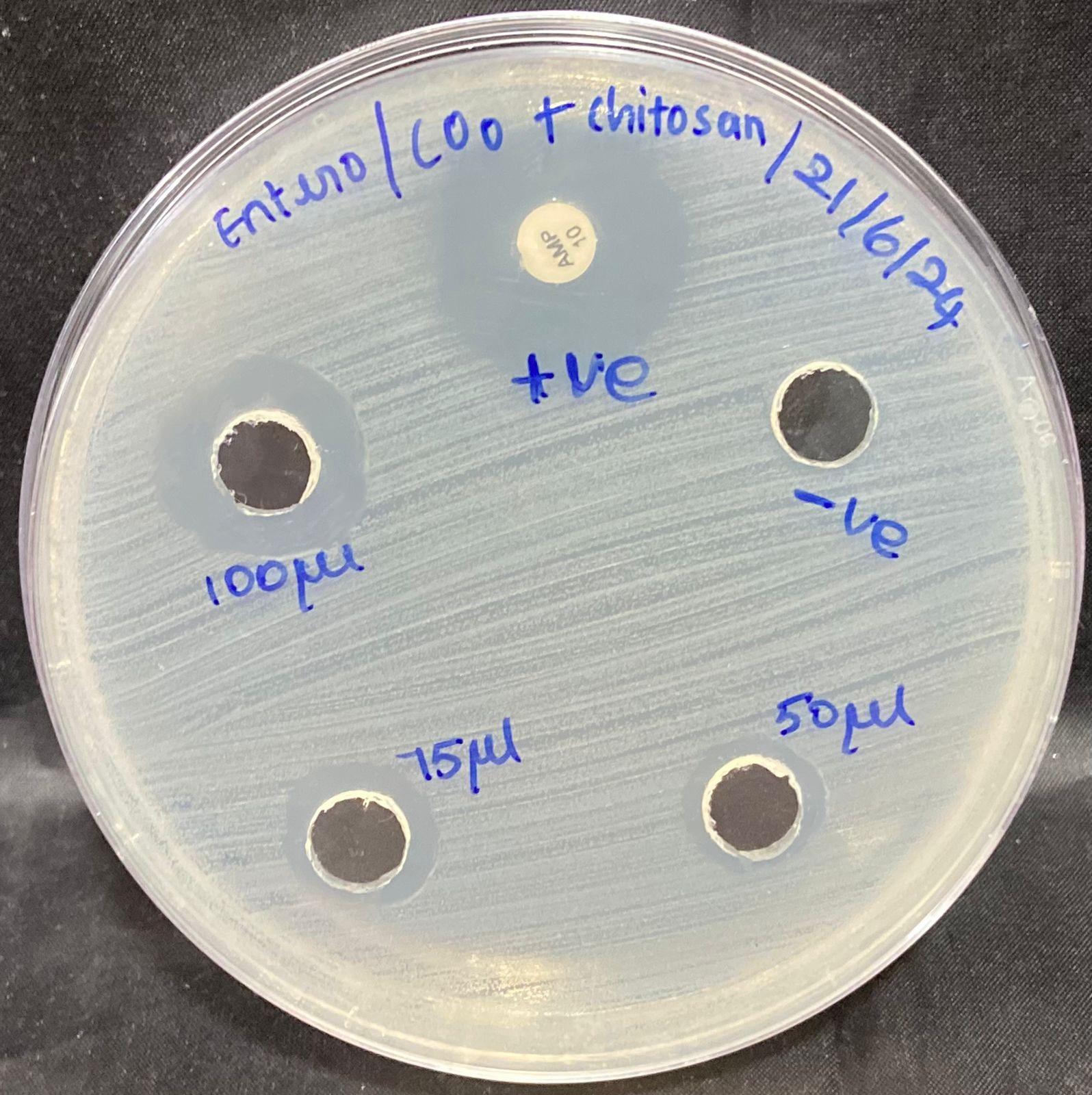
Fig.4 – (a) (b) (c) HRTEM imaging at magnifications of 200, and 100 nm respectively

## Antibacterial activity test

Antibacterial activity was tested using bacteria cultured on an agar plate. Gram-positive Enterococcus bacteria and gram-negative Escherichia coli bacteria were the two kinds of bacteria taken into consideration for this. A positive and negative control were incorporated for the purpose of comparison. The positive control used for Enterococcus was Ampicillin and for Escherichia coli it was Ciprofloxacin. The negative control used is known as DMSO solvent which is a polar aprotic solvent that possesses no antibacterial property. Concentration of synthesised CoO+Chitosan particles were varied to observe the antibacterial activity through measurement of the zone of inhibition. The area where no bacterial growth is seen is what we call a zone of inhibition. It is observed that a larger zone of inhibition is observed on higher concentration, however the increase is more prominent in E. coli compared to Enterococcus. On comparison at concentration 100 of CoO+Chitosan in **Fig.4** table, it can be observed that better antibacterial activity is observed in E.coli bacteria showing 17mm of inhibition compared to Enterococcus showing 14mm of inhibition.

Table 1: Antibacterial activity of cobalt oxide chitosan infused nanoparticles

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Concentration and zone of inhibition** | | | | **Positive Control** | **Negative Control** |
| **CS-CoO conc** | **50** | **75** | **100** | **Ampicillin and ciprofloxacin respectively** | **DMSO solvent** |
| **Enterococcus** | 11mm | 13mm | 14mm | 26mm | 10mm |
| **Escherichia coli** | 11mm | 13mm | 17mm | 23mm | 10mm |



1. (b)

Fig.5 – (a) (b) Antibacterial activity of CS-CoO NPs on Enterococcus and E.coli bacterial growth.

When compared to magnetic NPs without an appropriate surface coating, magnetic nanoparticles with the coating function better in biomedical applications [(Das et al., 2008)](https://paperpile.com/c/gMMe66/PDqb). Applying a benign substance, such chitosan, to the surface is a crucial strategy for surface modification. We used a chemical precipitation technique to create CS-CoO NPs [(24)](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4598679/). As seen in **Figure 2**, the most notable peaks that define chitosan nanoparticles are those of the hydroxyl and amine groups. This hydroxyl group (-OH) stretching vibration has a strong absorption at the wave-number 3627.92 cm-1 range. The C–H stretching vibration of the polymer backbone is demonstrated by the absorption peaks at 2981.73 and 2850.52 cm-1, respectively, at 1635.81 cm-1 (C–N stretching of amide I) and 1468.81 cm-1 (C–N stretching of amide II). The alkyl group's C–H bending vibration is the source of the strong peak at 1435.57 cm-1. In order to enhance the intra- and intermolecular contacts in chitosan NPs, the P=O linked together within the phosphoric bonds of TPP and the ammonium ions of the chitosan within these nanoparticles was shown to be the cause of the absorption at 1170.85 cm-1[(Dorozhkin, 2015)](https://paperpile.com/c/gMMe66/cYOw). The anti-symmetric vibrations brought on by the stretching of C–O–C bridges identify the absorption peaks at 1093.44 and 896.757 cm-1, which are ascribed to the glucopyranose ring in the chitosan matrix[(Peer et al., 2007)](https://paperpile.com/c/gMMe66/vsV5). According to the FTIR data shown in Figure 2, the amine groups, which shifted to 1726.66 and 1455.62 cm-1, and the hydroxyl groups, which also shifted to 3526.89 cm-1, were the two main functional groups in charge of the adsorption of Co2+

The delivery of anticancer or antibacterial agents is significantly influenced by the size of the nanocomplex because particles as small as 400 nm could readily migrate through the tumour tissues' weakened circulatory system before congregating within the microenvironment when lymphatic clearance is insufficient [(Sanpui et al., 2011)](https://paperpile.com/c/gMMe66/bL13). This phenomenon, referred to as "enhanced permeability and retention," serves as the foundation for "passive targeting" in the in vivo administration of antibacterial medications contained in polymeric nanocarriers. As a result, the chitosan infused cobalt oxide nanoparticles produced in this work fit the size requirement to be used as a medication delivery method[(Nair et al., 2022)](https://paperpile.com/c/gMMe66/NBQ9). Within this study it can be observed that the nanoparticles displayed significant antibacterial activity in combination with chitosan. Our study focuses on synthetic nanoparticles made from chitosan, however other studies have shown that chitosan-infused nanoparticles alone have shown a broad spectrum of antibacterial activity against gram-positive and gram-negative bacteria through various methods[(Chrzanowska et al., 2021)](https://paperpile.com/c/gMMe66/tNno) . Positively charged chitosan particles and charged negatively teichoic acid with peptidoglycans within gram-positive bacteria can interact electrostatically to rupture the cell membrane, release internal components, and let chitosan enter the microbial cells.

It has been demonstrated in earlier research that chitosan leads to the leakage of intracellular components, including proteins[(Sahariah & Másson, 2017)](https://paperpile.com/c/gMMe66/2X7i). Positively charged chitosan particles have the ability to damage gram-negative bacteria's outer membrane, allowing the bacterium to enter the cell and die. Positive charges from chitosan can balance out the high negative charges from lipopolysaccharide[(Yan et al., 2021)](https://paperpile.com/c/gMMe66/IyZ9) .

Studies have demonstrated that hydrolysis of peptidoglycans can lead to enhanced electrostatic connection; these findings are corroborated by assessments of the electric conductivity of the bacterial mixture and the inclusion of E.coli cytoplasmic b-galactosidase activities within the growth medium[(Feng et al., 2021)](https://paperpile.com/c/gMMe66/e4ek). Along with its antibacterial properties, chitosan may chelate with a large number of metal ions due to its significant affinity for them due to the abundance of amine and hydroxyl groups in it. Indeed, chitosan's antibacterial action can be increased by its ability to chelate metal ions. Previous reports have indicated that cobalt exhibits commendable antibacterial properties against both gram positive and gram negative bacteria, in addition to its cytotoxic effect against several cancer cell lines[(Chang et al., 2010)](https://paperpile.com/c/gMMe66/bkff) .

Another study examined how chitosan chelates cobalt's antibacterial properties worked against E.coli and Staphylococcus aureus, two common pathogens. They showed that the molar ratio of the metal ion was as important as the coordinated metal ion's characteristic in determining the inhibitory impact of the chelates. Unlike other bacteriostatic polymers, cobalt metal ions have distinct active sites, making them a significant class of antimicrobial agents. A research that focused on the chelation of chitosan with Co2+ and subsequent growth inhibitory studies against standard bacteria found that the impacts of the metal linkage to chitosan with ratio 1: 3 restrict the development of bacteria of Staphylococcus aureus and E. coli.[(Adewuyi et al., 2011)](https://paperpile.com/c/gMMe66/PTkL).

# Limitations

Concerns about cobalt oxide nanoparticle toxicity may arise based on the size, concentration, and length of exposure. Prior to any practical use, a thorough evaluation of their biocompatibility is essential, particularly in medical settings. It is necessary to address the stability and aggregation of nanoparticles in physiological circumstances to guarantee sustained antibacterial activity over time. It is still difficult to be sure that the antibacterial activity kills just pathogenic bacteria and leaves the good microbiota alone. Optimising their utilisation requires a thorough understanding of the precise mechanisms underlying the cobalt oxide nanoparticles and chitosan's synergistic antibacterial properties. For practical uses, scalable and affordable techniques of creating these hybrid materials must be developed.

# Conclusion

In this study, chitosan infused cobalt oxide particles were synthesised using the chemical precipitation method to pose an obstacle to antibiotic resistant bacteria. Gram positive and gram negative bacteria were both significantly inhibited by the nanoparticles. It is evident from the data that the gram negative bacteria exhibited a greater zone of inhibition and a more potent antibacterial action than the gram positive bacteria. This could be due to the fact that gram positive bacteria are known to have thicker cell walls. However, the results showed that both pathogens were significantly affected, suggesting that chitosan particles loaded with cobalt oxide nanoparticle ions may be a unique and highly dependable degradable key to suppress bacteria that are resistant to antibiotics. In order to improve formulation techniques and further investigate its effectiveness in successfully treating bacterial infections, future study should focus on comprehending the fundamental mechanisms of this synergy. These developments have great potential to solve issues in the environmental and healthcare fields, where antibiotic resistance is still a major worry.

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