Evaluation of Anti-Cancer Efficacy of a Novel Mouthwash Formulation Containing Calcium Oxide Nanoparticles and Chitosan Colloid

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**Abstract :**Advancements in nanoscience have significantly impacted oral healthcare, particularly in mouthwash development. Traditional mouthwashes often contain irritants and alcohol that can disrupt the oral microbiome. The study was undertaken to develop and evaluate safety and anticancer efficacy of a new mouthwash formulation based on a colloidal chitosan with calcium oxide nanoparticles (CaONPs).The mouthwash was prepared by mixing the sun-dried leaf powder of Atropa belladonna with water so that beneficial compounds are extracted from the plant material. Nanoparticles of calcium oxide developed, in a series of chemical reactions, by adding calcium nitrate, then adjusting the pH. Two formulations were screened- CHX with plant extract-based nanoparticles and CHX with plant extract, alone. Characterization techniques included fourier-transform infrared spectroscopy (FTIR), x-ray diffraction (XRD), and Scanning/Transmission electron microscopy (SEM/TEM).Characterization confirmed successful synthesis of CaO NPs, with FTIR identifying key functional groups and XRD revealing a crystalline structure. In vitro cytotoxicity tests on HeLa cancer cells demonstrated significant effects, with an 86% reduction in cell viability in a concentration of 100 μg/ml.These findings indicate a concentration-dependent cytotoxic effect, emphasising the importance of balancing antibacterial efficacy with the safety of oral tissues. Further research is essential to optimise the formulation for safe dental care applications.

**Keywords** Cytotoxicity, Novel mouthwash, Calcium Oxide Nanoparticles, Chitosan Colloid.

# Introduction

Developments in nanoscience and nanotechnologies across almost all scientific fields had made life easier in recent days. Because of the manner that atoms are structured on a scale of 1–100 nm, systems, devices, and structures in the fields of nanoscience and nanotechnology have unique properties and capacities. These are fast expanding fields of study [(Ramsundar et al., 2023; Rieshy et al., 2023; Singh et al., 2023)](https://paperpile.com/c/QgWIFa/W7eTI+xUN0p+hBqNE). The discipline experienced an increase in public discussion and knowledge around the beginning of the new century, which in turn cleared the path for the first applications of nanotechnology in commerce [(Rajeshkumar & Lakshmi, 2021; Sivakumar et al., 2021)](https://paperpile.com/c/QgWIFa/my2mg+U9NSf). Nanotechnologies are beneficial to almost every field of study, including physics, computer science, materials science, chemistry, biology, and engineering. Notably, remarkable advances in nanotechnology have been made in the field of human health recently, particularly in the area of cancer treatment [(Bayda et al., 2019)](https://paperpile.com/c/QgWIFa/33Bz).Mouthwashes are an essential component of everyday dental care regimens, and oral hygiene is vital to preserving general health[(Chokkattu et al., 2023; Dharman et al., 2023; Govindaraj & Shanmugam, 2023)](https://paperpile.com/c/QgWIFa/VSC8I+BO6eB+P5Grt). These products are frequently used to prevent gingivitis, lessen plaque, and control oral infections. Nevertheless, alcohol and other compounds found in many commercial mouthwashes have the potential to have negative consequences, including irritation of the mucosa and changes to the oral flora [(Pavithra et al., 2023; Shenoy et al., 2023; Thomas & Jain, 2023)](https://paperpile.com/c/QgWIFa/s9wo6+G6w3q+ERQv9). The development of mouthwashes using innovative materials that provide increased therapeutic effects and that are safe, biocompatible, and effective is gaining popularity [(Gandhi et al., 2021; Katyal et al., 2023; Priyadharshini et al., 2023)](https://paperpile.com/c/QgWIFa/NercP+18onu+V9eq4). Mouth rinses typically contain fluoride to prevent dental cavities and to stop microorganism growth [(Baehni & Takeuchi, 2003)](https://paperpile.com/c/QgWIFa/VblW). In situations such as post-subgingival scaling and root planing, in patients with poor oral hygiene, after periodontal surgery, and in patients who are physically or psychologically incompetent, mouth rinses are advised as an adjunct to mechanical dental care. The alcohol content of the mouthwashes on the market varies, with the maximum being 27% [(Bhatti et al., 1994)](https://paperpile.com/c/QgWIFa/d37V). The usage of alcohol-based mouth rinses has been linked to side effects, such as burning sensations in the mouth, ulcerations, oral mucosal hypersensitivity, and epithelium desquamation [(Gürgan et al., 2006)](https://paperpile.com/c/QgWIFa/IDPl).A therapeutic protein targets a certain cell type, and its activation results in its death. This end-point function is known as cytotoxicity [(Houde & Berkowitz, 2014)](https://paperpile.com/c/QgWIFa/AKtc). Cytotoxicity, or the capacity of certain medicinal proteins to cause cell death, is a crucial component of product safety and quality characteristics [(Egbuna et al., 2021)](https://paperpile.com/c/QgWIFa/oYye). Since cell growth has been disrupted, exposure to nanoparticles may have a harmful effect if it causes senescence but not death in cells [(Janani et al., 2021; Kachhara et al., 2021; Subramanian et al., 2023)](https://paperpile.com/c/QgWIFa/oQLUb+zat34+d9a7T). The quantity of cells available at various exposure times can be compared to reveal effects on cell growth [(Egbuna et al., 2021)](https://paperpile.com/c/QgWIFa/oYye). Developing a new mouthwash is called a novel mouthwash. Mouthwash solutions are designed to prevent the formation of biofilms, quorum sensing, and growth of bacteria on oral surfaces. The modes of action include toxicity, disruption of membranes, acidic and oxidative stress, disruption of protein translation, and interference with the metabolism of carbohydrates [(Doshi et al., 2023; Lampl et al., 2023; Pandiyan et al., 2023)](https://paperpile.com/c/QgWIFa/9lveE+DXgs3+pYJp4). In addition to eliminating germs, a mouthwash should also provide users fresh breath, numb pain, or promote healing in wounds by having an astringent effect, all without affecting salivary proteins or discoloration of teeth [(Bencze et al., 2023)](https://paperpile.com/c/QgWIFa/vHOQ).One thousand millionth of a metre is denoted by the Greek word "nano," meaning "dwarf" or "very small." It's critical to distinguish between nanoscience and nanotechnology[(Ali Mansoori, 2005)](https://paperpile.com/c/QgWIFa/nNVn). Nanotechnology is the engineering that uses this understanding to create real-world products like gadgets. Nanoscience is the study of structures and substances on nanoscales, which vary from 1 to 100 nm [(Gnach et al., 2015)](https://paperpile.com/c/QgWIFa/oTR6). Nanotechnology is regarded as one of the most interesting new technologies in the twenty-first century. It is the capability of controlling, quantifying, organizing, regulating, and/or manufacturing, at the nanometer level, everything required for building real-world, practical nanoscience-based products [(Allhoff et al., 2007)](https://paperpile.com/c/QgWIFa/WhKF). Being a natural polymer derivative of chitin, chitosan is attracting increasing attention due to spectacular biocompatibility, antibacterial action, and colloid-generating capacities. Nanoparticle dispersion chitosan colloids, therefore, turn out to be a great vehicle for ensuring their stability with continuous release. Chitosan, when used with CaO NPs in a mouthwash formulation, may have a synergistic effect enhancing antibacterial activity and other benefits which can be anti-inflammatory and wound healing [(Jana & Jana, 2020)](https://paperpile.com/c/QgWIFa/m8tH). What is more, duration of action of the mouthrinse can be further prolonged by chitosan because it forms a film on the oral mucosa [(Nagy et al., 2011)](https://paperpile.com/c/QgWIFa/I0PM). The most common way that chitosan kills bacteria is by adsorbing onto the negatively charged cell walls; it works from there by rupturing the membrane and changing the permeability of the substance. Later binding to the DNA halts DNA replication and eventually leads to cell death. Another possible theory is that chitosan behaves as a chelating agent, directly binding to metal ions to produce toxic compounds and thus would retard bacterial growth [(*Website*, n.d.)](https://paperpile.com/c/QgWIFa/ohvp).Given their exceptional antibacterial qualities and capacity to encourage the remineralization of tooth enamel, Calcium Oxide Nanoparticles, or CaO NPs, have become very promising agents in the field of oral healthcare. They have the ability to efficiently neutralise the acids that oral bacteria create, avoiding dental caries and improving oral health [(Nakamura et al., 2020)](https://paperpile.com/c/QgWIFa/4Gqc). Furthermore, because of their small size, they may interact and penetrate bacterial cells more effectively, improving the effectiveness of antibiotics. The use of CaO NPs in mouthwash formulations is still largely studied, despite these benefits[(Banik et al., 2025)](https://paperpile.com/c/QgWIFa/HTiP). The visual characteristics, antibacterial activity, and distinct crystalline structure of calcium oxide nanoparticles are noteworthy [(Kawashima et al., 2009)](https://paperpile.com/c/QgWIFa/zMQ1). They are found in many industries as catalysts and remediation agents for hazardous waste, which helps to protect the environment [(Osuntokun et al., 2018)](https://paperpile.com/c/QgWIFa/rpDE). Functional groups are included in the structure of chitosan, which gives rise to a number of properties such as low toxicity, being biodegradable and cancer prevention activity [(Arjunan et al., 2017)](https://paperpile.com/c/QgWIFa/hhYt). Due to chelating properties, chitosan can interact with metal oxides to affect dyes, biosensors, and hazardous organic compounds. Chitosan is a hydrophilic polymer containing NH2 and OH side groups that can form hydrogen bonds with calcium oxide nanoparticles to enclose them and create special nanocomposites [(Deshmukh & Pandey, 2023)](https://paperpile.com/c/QgWIFa/evWX).Therefore the aim of this study is to create a novel mouthwash with a nanocomposite complex and analyse the change in cytotoxicity of the created mouthwash. The purpose of this study is to assess the cytotoxicity and characteristics of a new mouthwash that contains chitosan colloid and calcium oxide nanoparticles. Through methodical examination of this formulation's physicochemical characteristics, antibacterial efficaciousness, and cytotoxicity, we want to ascertain its potential as a secure and efficient oral care product.

# Materials and Method

## Preparation of plant extract

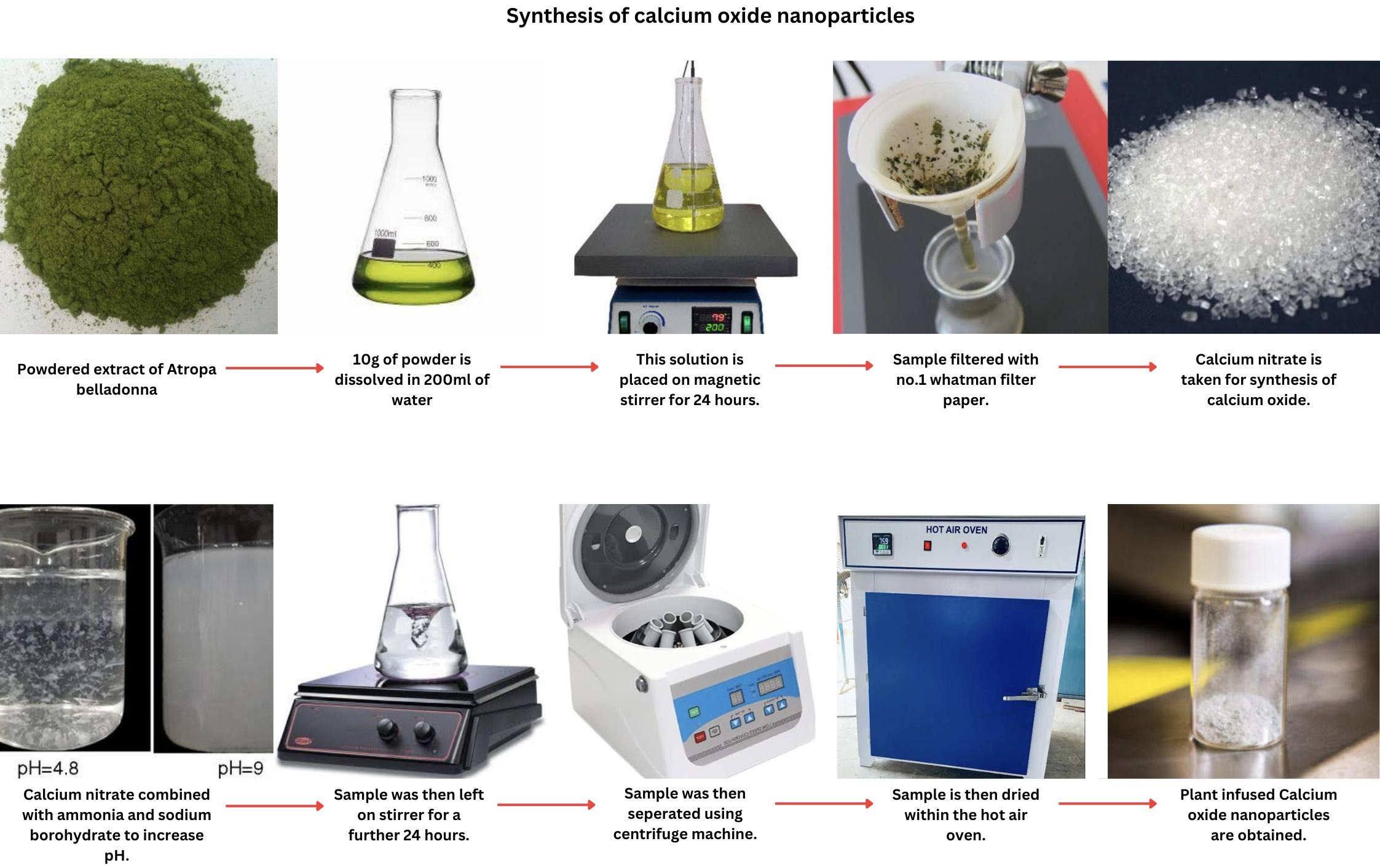
The leaves from Atropa belladonna were collected from several places within Subsequently after removal of undesired substances using distilled water, samples were shade dried to preserve true originality. The leaves were ground into a fine powder by hand with a mortar and pestle once they had fully dried in the shade. Next, 200 ml of distilled water and 10 g of powdered leaf material were mixed together in a sterile conical flask. For a full day, the mixture was heated and agitated. The resulting extract was then filtered to remove contaminants using Whatman No. 1 filter paper. [(Sasidharan et al., 2010)](https://paperpile.com/c/QgWIFa/IOe6).

## Preparation of calcium oxide nanoparticles

Initially, distilled water was added to a precisely calibrated solution of Calcium nitrate (0.4 M, along with the filtrate obtained from the plant extract. The mixture was boiled while stirring with a magnetic agitator. At the same time 1 M sodium borohydride solution and ammonia solution were gradually added until the characteristic blackish / brown color was produced (indicating production of CaO NPs). For fifteen minutes, the mixture was centrifuged at 5,000 rpm. The pellet and supernatant were separated following centrifugation. After discarding the supernatant liquid, the pellet containing the nanoparticles was progressively moved into a petri plate. Further dried the nanoparticles at 100°C in a hot air oven. The properties of the CaO NPs produced with A. belladonna extract have been studied extensively.

## Preparation of mouthwash

Two types of mouthwash were made for this study (double distilled water and chlorhexidine gluconate (CHX) as principal solvent. The principal solvent first used was a mixture of plant derived nanoparticles with 0. 2% CHX. How they did it was make a mouthwash just with the plant extract with 0. 2% CHX This was done so that comparison and analysis of the effect could be done on effects of nanoparticles on the antibacterial effect of the developed mouthwash.



**Fig 1** - Synthesis of calcium oxide nanoparticles

## Characterization

Utilizing a Bruker Alpha-II ATR equipment, spectral interpretation utilizing Fourier-transform infrared (FTIR) spectroscopy was carried out within the wavelength range of 4000–400 cm-1. Using Eu Kα radiation, XRD analysis was performed on the finely ground CaO NPs to look into their crystal structure. The D8 Advance X-ray diffractometer from Bruker was used to interpret the spectrum at 30 kV and 15 mA. Diffraction intensities were measured throughout a range of 2θ angles, from 20 to 80 °, at a rate of 4°/min and a step size of 0.05°. A Jeol JSM IT 800 instrument was used for SEM analysis, while a G2 20 S - Twin TEM apparatus was used for TEM examination.

## Anticancer activity

The MTT assay was used to assess the synthesized CaO NPs' in vitro anticancer efficacy against MCF-7 breast cancer cell lines. HeLa cells were cultivated in 96-well plates after proper cultivation in appropriate growth medium. Control samples and various concentrations of CaO NPs were added to several wells. MTT reagent was added for incubation, which helped in the formation of formazan crystals. Absorbance readings were recorded to ascertain cell vitality. The IC50 was calculated, which inhibited 50% of the cell viability at a concentration. This approach provides an in-depth assessment of the anticancer features of CaO NPs in HeLa cancer cells, showing lethal and therapeutic features.

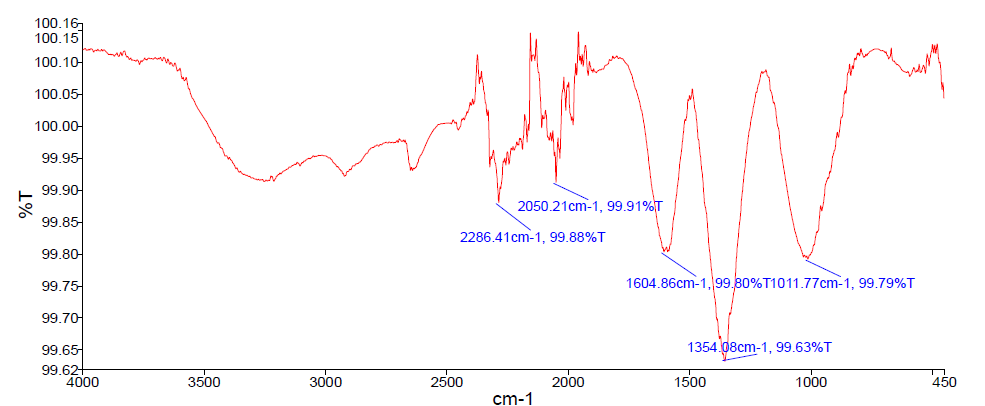
# Statistical analysis

To ensure reliability, the tests are normally run three times. The collected data is often expressed as mean values with comparable standard deviations. To produce the IC50 value, or the concentration at which 50% inhibition occurred (i. e. it was accurately attributed and proved precisely with the same rigor) in the statistical analysis of ANOVA and linear regression model an important metric to assess the biological activity and probable applications of nanoparticles IC50 value (Almatrafi et al., 2024).

# Results

## Fourier-Transform Infrared (FTIR) spectroscopy

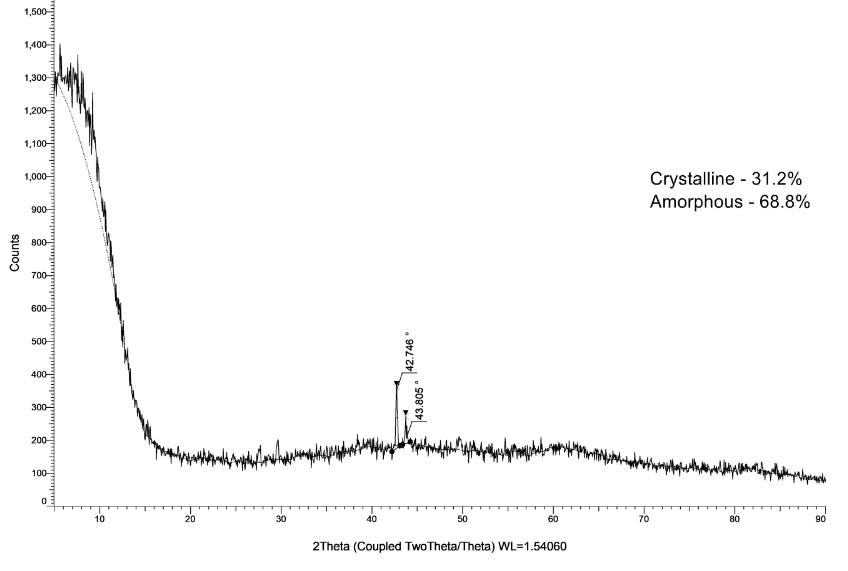
Fourier-transform infrared (FTIR) spectroscopy (FTIR) has been used to characterise calcium oxide (CaO) nanoparticles synthesised by Atropa belladonna leaf extract and many functional groups in the nanoparticle formation process have been demonstrated(Saadh et al., 2024). There were notable peaks at 2286.41 cm⁻¹, 2050.21 cm⁻¹, 1604.86 cm⁻¹, 1354.08 cm⁻¹, and 1011.71 cm⁻¹ in the FTIR spectrum. Nitriles may be present since the peak at 2286.41 cm⁻¹ is linked to C–N stretching vibrations. Alkyne groups are indicated by the peak at 2050.21 cm⁻¹, which corresponds to C≡C stretching vibrations. C=C stretching vibrations, which may originate from aromatic rings or alkenes found in the leaf extract, are responsible for the peak at 1604.86 cm⁻¹. C-H bending vibrations are represented by the peak at 1354.08 cm⁻¹, whereas C-O stretching vibrations—likely from alcohols or ethers—are associated with the peak at 1011.71 cm⁻¹. During the synthesis process, these functional groups play a critical role in a decrease and stabilisation of calcium ions.



**Fig.2** : FTIR Spectroscopy has provided valuable insights into the composition of the synthesised CaO nanoparticles

## X-ray diffraction (XRD)

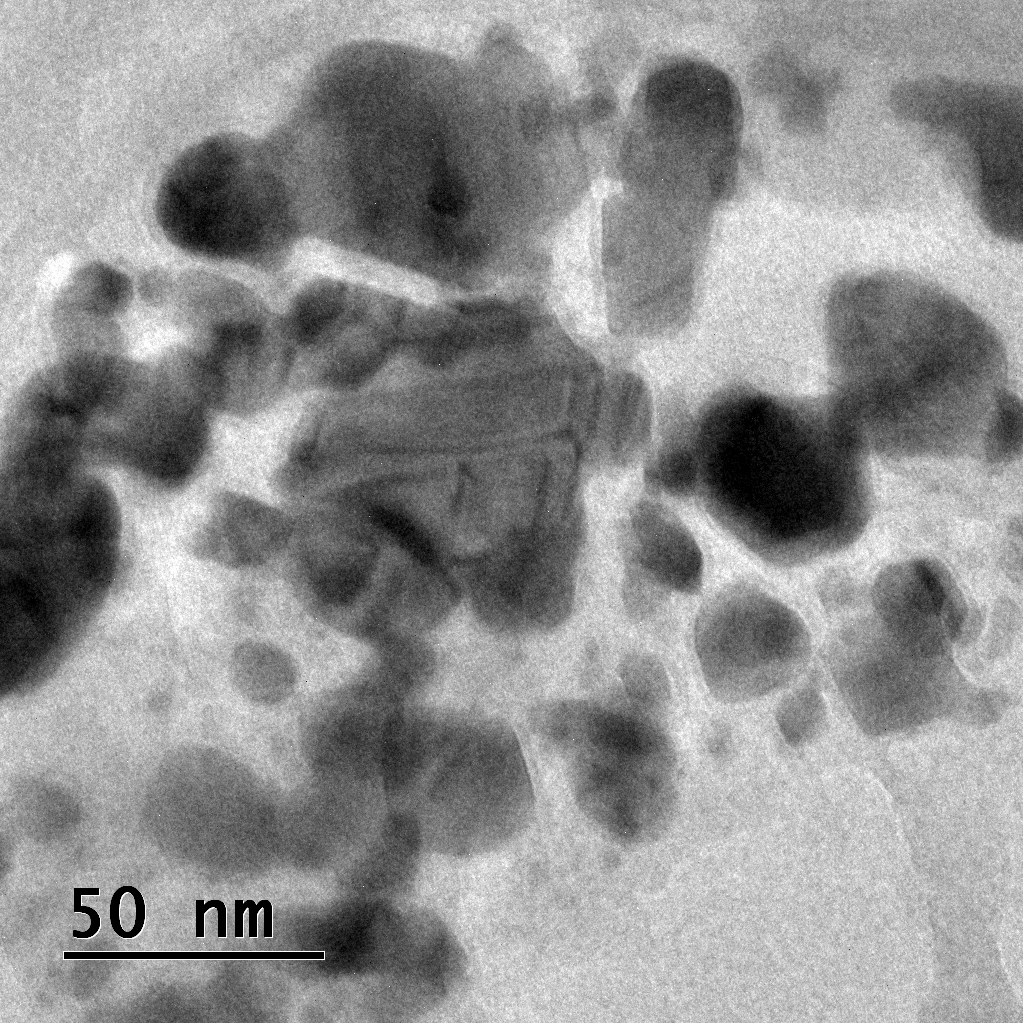
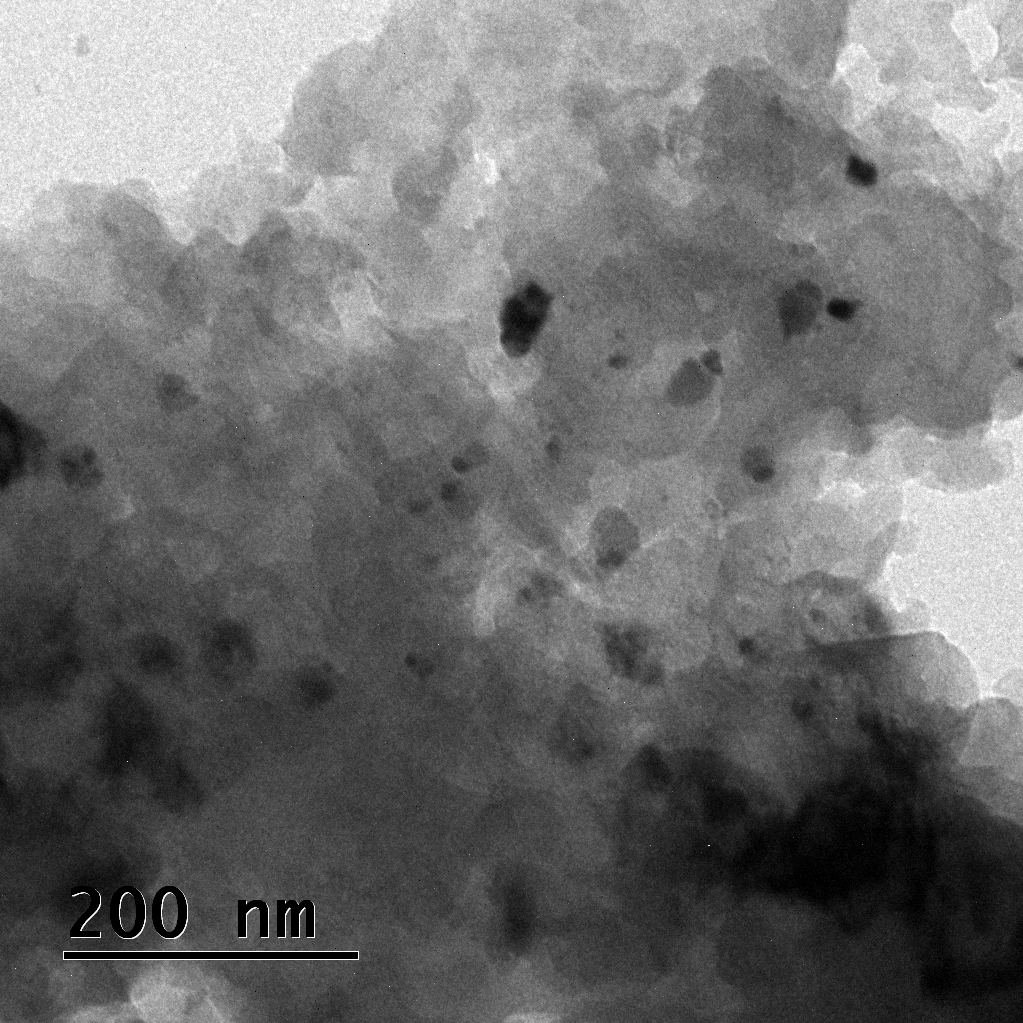
X-ray Diffraction (XRD) was used to characterise calcium oxide (CaO) nanoparticles that were synthesised using Atropa belladonna leaf extract. The results showed different crystalline characteristics. The XRD pattern revealed notable peaks at 42.74° and 43.80°, suggesting that the CaO nanoparticles are crystalline. Standard crystallographic data identifies these peaks as corresponding to particular lattice planes within the CaO crystal structure. The creation of well-crystallised CaO nanoparticles is confirmed by the peak at 43.80°, which correlates to the (202) plane and is typically linked with the (200) plane.



**Fig.3** X-ray diffraction (XRD) analysis confirmed crystalline calcium oxide (CaO) nanoparticles

## Transmission Electron Microscopy (TEM)

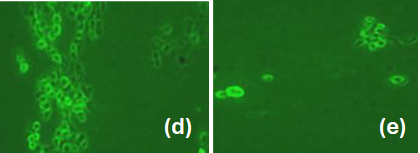
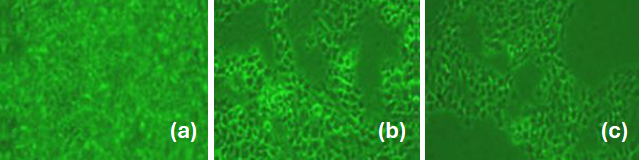
The morphology of calcium oxide nanoparticles were examined using High-Resolution Transmission Electron Microscopy (HRTEM), which showed that the NPs were spherical and had a mean size of 42 nm. Prior to imaging, the sample was dried to remove solvent interference and guarantee that the NPs could be seen clearly. Their structural properties could be observed and their size could be accurately measured thanks to this drying process. The outcomes, which are shown in Fig. 4, show how uniform the nanoparticles are. These qualities are essential for their possible uses in material science and catalysis, for example. All things considered, HRTEM is necessary for thorough nanoparticle characterisation.



**Fig.4** - TEM analysis showing particle size, shape and distribution. (Crystalline particles with a median diameter of 42 nm on average.)

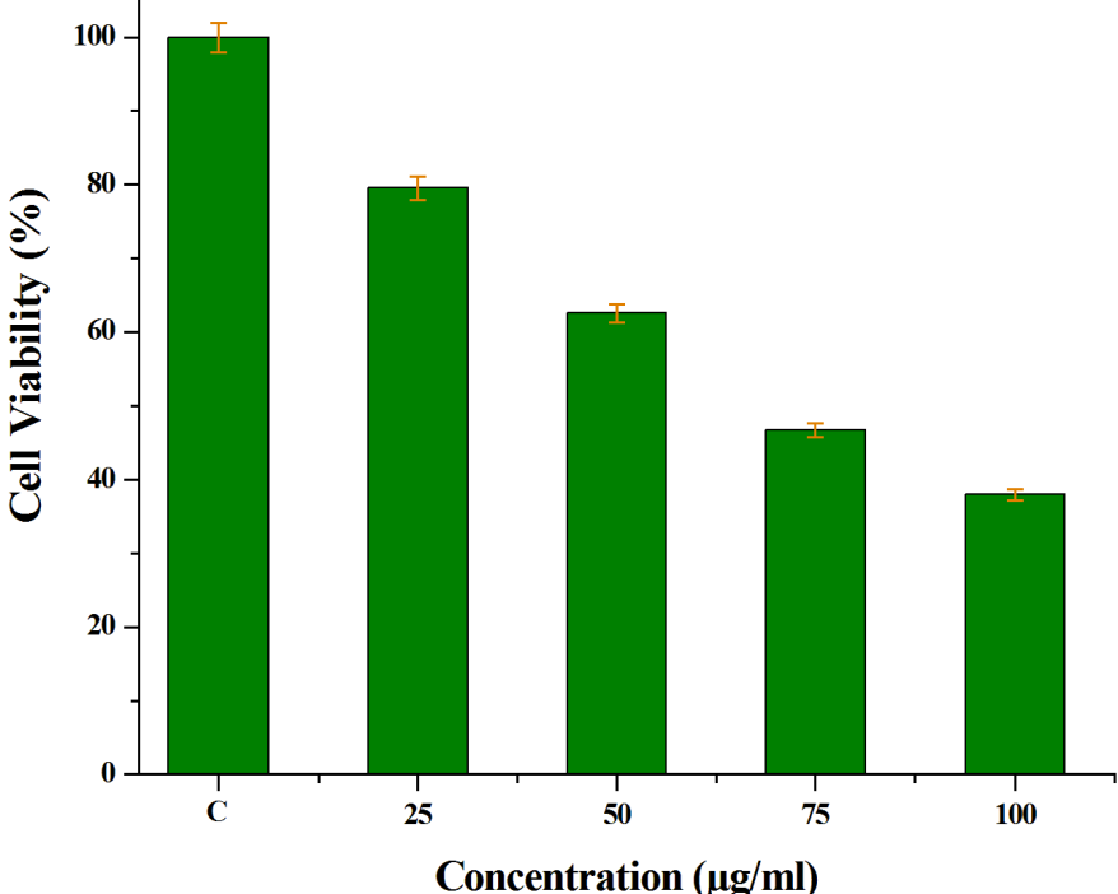
# Anticancer activity

At varying doses (25-100 μg/ml), the anticancer properties of calcium oxide nanoparticles were assessed in vitro against HeLa cancer cell lines (**Fig. 5**). In the examined cell lines, the synthesised nanoparticles showed the strongest cytotoxic action. On observation in **Fig. 6**, the results demonstrated that cell viability declined with increasing doses. After incubation for twenty-four hours, the inhibition effect was noticed. At a dosage of 100 μg/ml, the synthesised nanoparticles activated 86% of HeLa cancer cells. The HeLa cells' viability was able to be decreased by the synthesised nanoparticles in a dose-dependent way. It was discovered that the substance was a strong cytotoxic agent against the HeLa cell line. As a result, the produced nanoparticles might be regarded as a potent anticancer medication.



**Fig.5 –** (a) (e)Cytotoxic activities of calcium oxide nanoparticles HeLa cell lines at various concentrations (25 μg/ml [b], 50 μg/ml [c], 75 μg/ml [d], 100 μg/ml [e]) with control [a].

## Cytotoxic activity



**Fig.6** The % of cell viability of calcium oxide nanoparticles against HeLa cell lines at various concentrations (25-100 μg/ml) with control (C).

# Discussion

It is evident from the results of the characterisation analysis tests conducted on the sample that the investigation has made great progress. When Fourier-transform infrared spectroscopy (FTIR) is examined, it reveals distinct peaks that stand in for the functional groups that enable calcium ion stabilisation. The aqueous extract of Atropa belladonna contains a wide range of phytochemicals, such as alkaloids, glycosides, phenols, tannins, saponins, glucosinolate, and flavonoids. These primary bioactive constituents can be reduced from precursors of calcium metal salts (Ca2+) to CaO NPs and will function as capping and stabilising agents. Figure 2 FTIR spectroscopy is used to confirm the development of the CaO NPs crystal structure. CaO NP production was confirmed by the prominent infrared band at 534 cm-1, which was attributed to fundamental vibrations of CaO [(Akbar, 2020)](https://paperpile.com/c/QgWIFa/nxmv) X-ray Diffraction analysis (XRD) shows significant peaks which show that the calcium oxide nanoparticles have crystalline form. Fig. 5 shows the cytotoxic effect of calcium oxide nanoparticles in various concentrations relative to the control shows that calcium oxide nanoparticles showed an increased antibacterial activity against HeLa cell lines in particular at 100g/ml. The fact that Fig. 6 shows a significant decrease in the cell viability percentage of HeLa cell lines in the presence of higher concentrations of calcium oxide nanoparticles shows that the synthesised particles are capable of having strong cytotoxic activity. The new mouthwash comprising CaO nanoparticles was evaluated for cytotoxicity, and the results indicated concentration-dependent impacts on cell survival[(S et al., 2023)](https://paperpile.com/c/QgWIFa/vmGB). Lower amounts suggested less cytotoxicity since they preserved vitality comparable to untreated cells. On the other hand, viability dramatically dropped at greater concentrations, suggesting strong cytotoxic effects. The cytotoxicity of dental care products was shown to be within permissible limits through comparative study with positive controls [(de Souza et al., 2020)](https://paperpile.com/c/QgWIFa/jAXo).It is well known that calcium oxide has strong antibacterial, antioxidant, and biodegradable qualities. The stabilising agent utilised in calcium oxide nanoparticles is chitosan colloid since the particles are extremely unstable at zero oxidation state. This study discovered that a mouthwash made of chitosan colloid and calcium oxide nanoparticles has potent antibacterial qualities [(Sreenivasan et al., 2004)](https://paperpile.com/c/QgWIFa/z629). In addition to being proven to have potent antibacterial qualities, the herbal calcium oxide nanoparticle mouthwash is alcohol-free. Research has demonstrated that individuals who are susceptible to ulceration and mucositis should not use mouthwashes containing alcohol. Additionally, a link was shown between the development of oral cancer and the usage of mouthwashes containing alcohol. This reinforces the need to refrain from using mouthwashes with alcohol [(Winn et al., 1991)](https://paperpile.com/c/QgWIFa/vEaO).

# Conclusion

In conclusion, our cytotoxicity analysis of the new mouthwash with calcium oxide nanoparticles shows impacts on cell viability that are concentration-dependent. Lower concentrations show negligible cytotoxicity that is comparable to untreated cells, indicating that these amounts may be safe. Higher doses, however, have noticeable cytotoxic effects, emphasising the necessity for cautious formulation optimization to strike a balance between oral mucosal safety and antibacterial efficacy. To make the mouthwash more effective and compliant with strict safety regulations so that it can be used in dental care, more research is necessary.

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