

# Biosynthesis of Calcium Oxide Nanoparticles using *Commelina Benghalensis* and its Cytotoxic and Embryonic Toxicology Evaluation

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Synthesis of non-toxic nanoparticles through biosynthesis has become a necessity in the last few years due to increased reports of drug toxicity. Biosynthesis with an appropriate plant species is important to ensure that the plant's phytochemicals do not negatively affect the properties of the drug. This study was aimed to analyze the cytotoxic and embryological toxic properties of *Commelina benghalensis*-mediated calcium oxide nanoparticles and their potential applications. Leaves of *Commelina benghalensis* were powdered and boiled in the precursor to synthesize the calcium oxide nanoparticles. The nanoparticles were characterized by an UV-visible spectrophotometer and their embryological toxicity and cytotoxicity were evaluated through zebrafish embryological assay and brine shrimp lethality assay respectively. The green synthesized nanoparticles were analyzed using UV-visible spectra showing the characteristic peak at 295 nm and then their cytotoxic and toxicity were analyzed through brine shrimps and zebrafish embryos. It was found that the green synthesized calcium oxide nanoparticles had none to negligible effect on the test organisms up to 40 µg/ml. Noticeable effects were seen only at 80 µg/ml with a very low difference compared to control. The small difference to the standard proves that *Commelina benghalensis*-mediated Calcium oxide nanoparticles have a great scope as potential drug delivery agents. Safe and controlled animal testing can be conducted before clinical trials of these nanoparticles in patients.

**Keywords:** Calcium oxide nanoparticles, *Commelina benghalensis*, cytotoxic effect, green synthesis, zebrafish embryonic toxicology.

## 1. Introduction

Nanotechnology is the field of biomedicine which deals with synthesis and characterization of

the nanoparticles (Marquis et al., 2016) Over the past few years, application of nanoparticles has increased extensively especially in the fields of public health. They showcase unique physicochemical properties like catalytic activity, antibacterial properties, high surface area ratio etc (Roy et al., 2013). Combining nanotechnology with biology opens a new door for the development of nanosized materials which could see endless utilization in healthcare. With the right synthesis and stabilizing agents, the nanoparticles are made biocompatible (both nonimmunogenic and nonantigenic) (Kango et al., 2013). Based on their size, morphology, reactivity and stability, NPs show excellent properties in comparison to the bulk compound (Khan et al., 2019). Nanoparticles provide space and can protect the integrity of drugs during circulation in blood and prevent interaction between drug molecules and non-target tissues (Min et al., 2023).

Calcium oxide (CaO) has been widely used for disinfection of multiple pathogens especially during epidemics (Hata et al., 2021). CaO is a photoactive compound which degrades pollutants and also a potential heterogeneous catalyst which is also eco-friendly (Alsohaimi et al., 2020). CaO nanoparticles (NPs) have a great scope as an easily obtainable compound with antibiofilm, antifungal, antibacterial and antioxidant properties (Kumari et al., 2023). The antibacterial activity of CaONPs, often attributed to the superoxide and peroxide free radicals, allows the NPs to destroy the bacterial wall and create an antioxidant imbalance in the microbes. CaONPs can be prepared by multiple ways including microwave processes, thermal decomposition, and water-oil microemulsions. But all of these methods have certain limitations such as high temperature requirements, expensive apparatus, probable toxicity and prolonged process time (Atchudan et al., 2022).

Biosynthesis or green synthesis of nanoparticles is an environmentally friendly way of producing NPs while minimizing the waste produced chemical involvement and toxicity prevalence. This alternative has been gaining importance over the last decade, especially in pharmaceuticals (Harris et al., 2023). Nanoparticles generated through the green synthesis have a diverse nature, offer superior stability, and suitable dimensions due to the use of a one-step process for its synthesis (Maringgal et al., 2020). It is well known that the phytochemicals in plants play a major role in reduction during oxide production (Jadhav et al., 2022). For this study, *Commelina benghalensis*, also known as the Benghal Dayflower or Tropical Spiderwort was utilized for green synthesis. It is commonly found in the tropical regions of Asia and Africa. The plant has widespread applications in conditions like constipation, pain, cataract, jaundice, scabies and mental illness and many others. Different parts of the plant contain flavonoids, tannins, quinones, coumarins and lactones all of which play a role in its medicinal usage (Roy Orni et al., 2018).

Nanoparticle based drugs have to undergo cytotoxicity and embryonic toxicity screening to identify potential adverse effects (Fröhlich et al., 2011). Eliminating the possibility of both cytotoxicity and embryological toxicity is necessary in making a compound highly effective while being safe to use. The aim of this study is to evaluate the cytotoxicity and embryonic toxicity of *C. benghalensis*-mediated, green synthesized calcium oxide nanoparticles through brine shrimp lethality assay and Zebrafish embryonic toxicology evaluation respectively.

## 2. Materials and Methods

### 2.1. Preparation of plant extract

Fresh *Commelina benghalensis* leaves, collected from the herbal garden of Saveetha medical college, were shade dried and powdered. 1 gram of *Commelina benghalensis* plant powder was added to 100 mL of distilled water. The plant extract was then boiled at 50-60°C using a heating mantle for 10 minutes. Later, the boiled extract was filtered using a muslin cloth. 50 mL of the filtered plant extract was then added to 50 mL of precursor solution to form 100 mL of resultant solution.

### 2.2. Preparation of nanoparticles

30 millimolar of calcium hydroxide [ $\text{Ca}(\text{OH})_2$ ] was mixed in 50 mL of distilled water. *C. benghalensis* acts as the reducing and stabilizing agent. Calcium hydroxide solution was used as the precursor solution. The resultant 100 mL solution was kept in a magnetic stirrer for 48 hours for synthesis of nanoparticles to occur. The magnetic stirrer was checked after the first 24 hours to confirm a change in color of the solution which is the preliminary confirmation for nanoparticle synthesis. After 48 hours, the nanoparticle solution was centrifuged at 8000 rpm (rotation per minute) for 10 minutes. After this procedure, the pellet was collected and kept in the refrigerator for further use. The synthesized nanoparticles were preliminarily characterized using a UV-visible spectrophotometer and the nanoparticles showed the characteristic peak at 250-650 nm.

### 2.3. Brine shrimp lethality assay

The cytotoxic effect of CaONPs was assessed using Brine shrimp lethality assay. 2 grams of salt (iodine-free) was weighed before dissolving it in 200 milliliters of distilled water thoroughly. 6 well ELISA plates were taken and filled with 10-12 ml of saline water. Subsequently, 10 nauplii were carefully introduced into each well (5, 10, 20, 40, and 80  $\mu\text{g/mL}$ ). Following that, calcium oxide nanoparticles were incorporated based on the respective concentration levels. The plates were then subjected to an incubation period of 24 hours. ELISA plates were examined after 24 hours of incubation and the number of viable nauplii was recorded. The percentage of live nauplii was calculated using the following formula:  $\text{No. of dead nauplii} / \text{No. of dead nauplii} + \text{No. of live nauplii} \times 100$

### 2.4. Zebrafish embryonic toxicology evaluation of calcium oxide nanoparticles

The cytotoxic effect of CaONPs was evaluated using zebrafish embryonic toxicology analysis. Wild variety of Zebrafish (*Danio rerio*) were Sourced from indigenous Indian suppliers and kept in separate tanks with optimal temperature conditions ( $280 \pm 20^\circ\text{C}$ ), light/dark cycle (14:10 h), and pH (6.8 - 8.5). Commercially available dry blood worms and optimum food was fed to the embryos every day. The embryos were obtained by mating a single female with three males in a breeding tank. Following that, viable eggs were carefully collected and freshly prepared E3 medium without methylene blue was used to wash them at least three times. The research involved placing fertilized eggs in culture plates of different well sizes (6, 12, and 24 wells) with 20 embryos per 2 mL solution per well. The experimental treatment and control groups were replicated three times. The experimental treatment was formulated by freshly preparing a stock suspension of TCF-CaONPs with all five concentrations, which were subsequently combined with the E3 medium. Following this, the solution underwent sonication for 15

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minutes to disperse the nanoparticles evenly, with careful monitoring to maintain a pH level between 7.2-7.3. The healthy fertilized embryos were subjected to different levels of CaONPs, with concentrations varying from 5 to 80  $\mu\text{g/mL}$ , for a period of 24 to 96 hours following fertilization. The embryos were placed in the E3 medium, supplemented with the CaONPs, for incubation. Control groups were incorporated into the research design. Deceased embryos in the nanoparticle-induced groups were eliminated every 12 hours. To ensure light exclusion, all the experimental plates were maintained at a temperature of 28°C after wrapping them in foil.

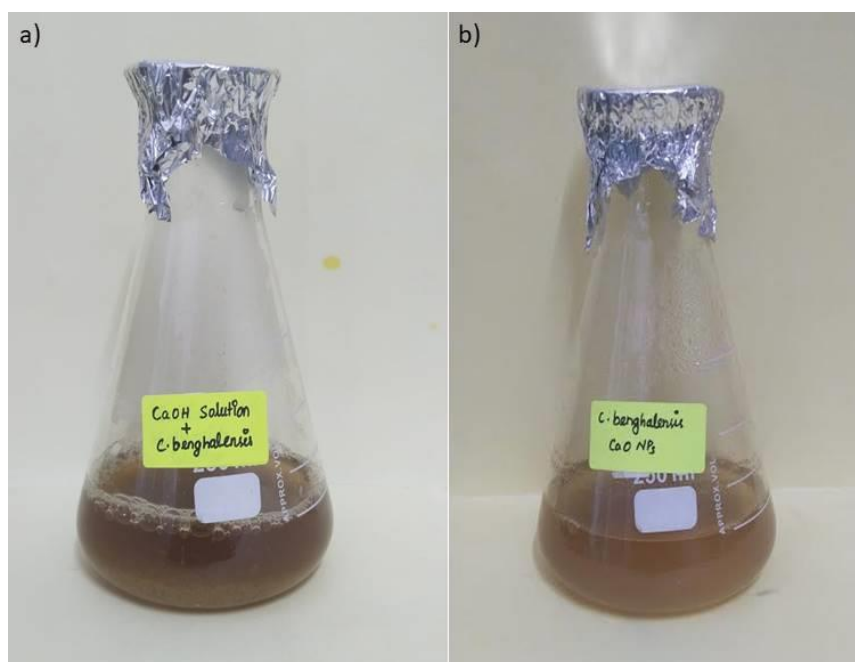
The developmental stages of Zebrafish embryos were monitored with a stereo microscope throughout the exposure period post-fertilization. These embryos were then treated with varying concentrations of CaONPs (5, 10, 20, 40, and 80  $\mu\text{g/mL}$ ) for 24-78 hpf. The study assessed embryo/hatchling mortality, hatching rate, and the presence of malformations in both control and test groups. The defective embryos were imaged using a COSLAB - Model: HL-10A light microscope. The ratio of abnormal embryos was documented once every 24 hours.

### 3. Results and Discussion

#### 3.1. Visual observation

The colour of the *Commelina benghalensis* plant extract mediated calcium oxide nanoparticles solution was changed from dark brown to light brown in colour after 24 hours as shown in figure 1 which preliminarily confirms the preparation of nanoparticles.

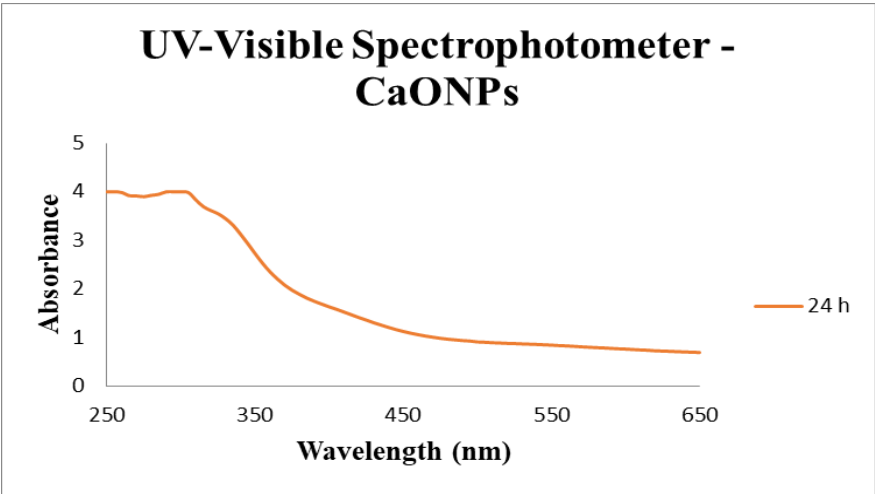
Figure 1: Visual observation of *Commelina benghalensis* plant extract mediated calcium oxide nanoparticles a) Initial colour change b) final colour change



3.2. UV-visible spectroscopy

The absorbance of the *Commelina benghalensis* plant extract mediated calcium oxide nanoparticles was observed between 250-650 nm and an absorbance peak was obtained at 295 nm as displayed in figure 2 which preliminary confirms the presence of calcium oxide nanoparticles.

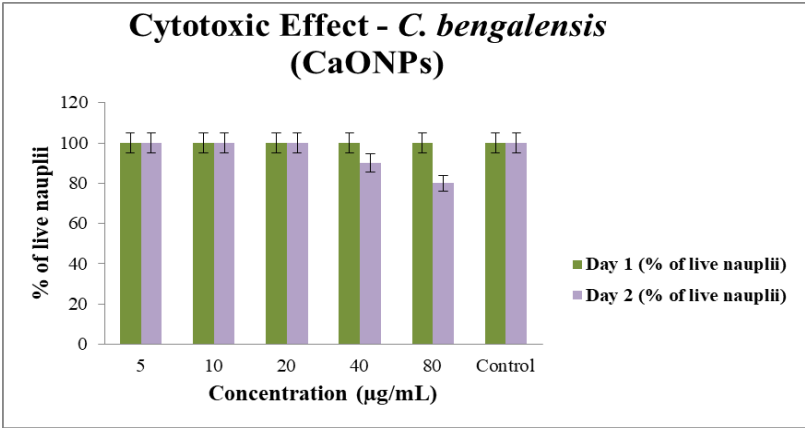
Figure 2: Graphical representation of UV-visible spectra of calcium oxide nanoparticles.



3.3. Brine shrimp lethality assay

Larvae of shrimps, called nauplii, were utilized to evaluate the cytotoxic effects. 60 nauplii were equally distributed into five different wells with concentrations of 5, 10, 20, 40 and 80 µg/ml and one control well. The effects of the *Commelina benghalensis*-mediated calcium oxide nanoparticles were observed in 24 hour intervals for 2 days. As seen in figure 3, all nauplii were alive over the two days in the 5, 10 and 20 µg/ml wells. On the second day, in the 40 and 80 µg/ml wells, 90% and 80% of nauplii were alive respectively.

Figure 3: Graphical representation of cytotoxicity of *Commelina benghalensis*-mediated Calcium oxide nanoparticles using brine shrimps.



### 3.4. Embryonic toxicology

Zebrafish embryos were first treated with varying concentrations of the extract and their developmental changes were observed. Viability of the test and control groups of embryos was shown in figure 4, 5 and 6. No significant change was observed at concentrations of 5, 10 and 20  $\mu\text{g/ml}$ . But increase in concentration to 40 and 80  $\mu\text{g/ml}$  lead to a significant effect on the mortality of the embryos. Figure 6 displays the rate of successful hatching of zebrafish embryos. At 5 and 10  $\mu\text{g/ml}$ , the embryos remained unaffected. About 80% hatching rate was seen at 20 and 40  $\mu\text{g/ml}$ . On increasing the concentration to 80  $\mu\text{g/ml}$ , more than 40% of embryos were unable to hatch.

Figure 4: Microscopic images of wild zebrafish embryo treated with *Commelina benghalensis*-mediated Calcium oxide nanoparticles (a) Day 1 (b) Day 2 (c) Day 3.

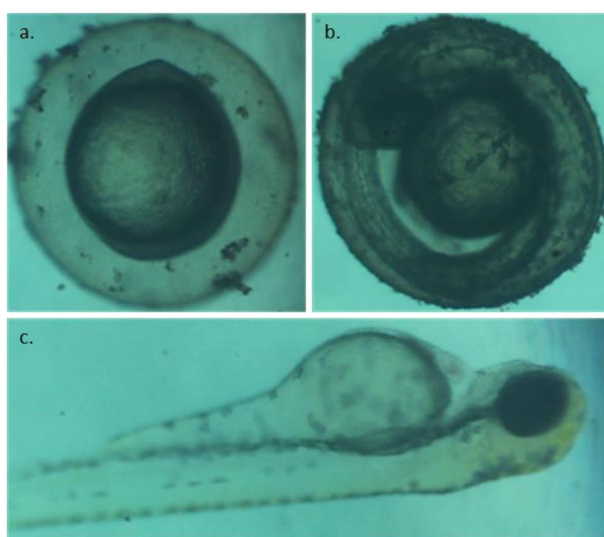


Figure 5: Graphical representation of the viability rate of wild zebrafish on treatment with *Commelina benghalensis*-mediated Calcium oxide nanoparticles.

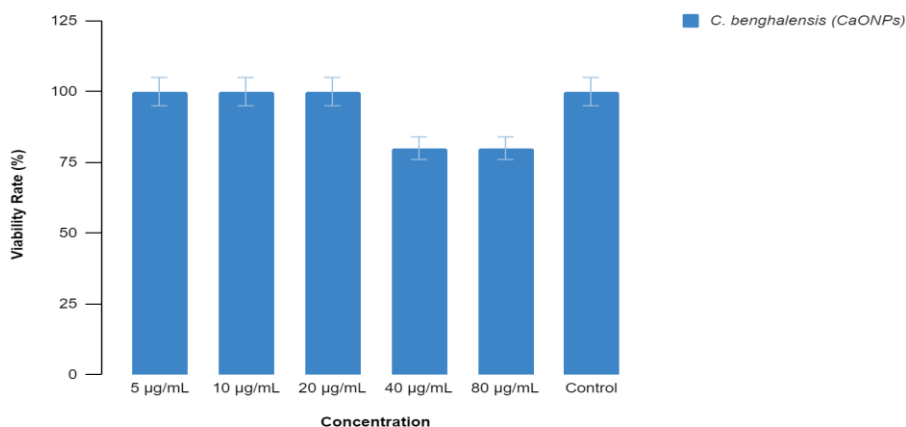
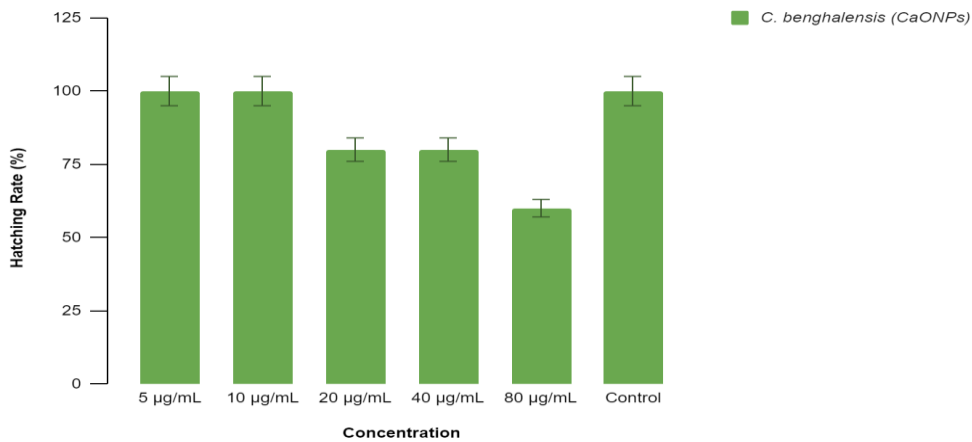


Figure 6: Graphical representation of the hatching rate of wild zebrafish on treatment with *Commelina benghalensis*-mediated Calcium oxide nanoparticles.



#### 4. Discussion

Nanotechnology has been gaining importance over the last decade. This branch of science has been utilized to overcome multiple obstacles faced by researchers and scientists for centuries. With the need of nanoparticles increasing in various fields, extensive research on their properties and applications has become prominent. The method of synthesizing is a crucial part of every study as it determines the properties of the nanoparticles formed. For this study, we have green synthesized the nanoparticles using *Commelina benghalensis*. The plant is commonly known as the Bengal dayflower, and has been explored for its potential in synthesizing CaONPs due to its rich phytochemical composition.

Calcium oxide nanoparticles were successfully synthesized with *Commelina benghalensis* mediation. According to another study where neem leaves were utilized for green synthesis for calcium oxide nanoparticles (Mazher et al., 2023), green synthesis imparts beneficial properties of the plant while enhancing the natural properties of the nanoparticles. Similar to another study where *Azadirachta indica* was used for calcium oxide nanoparticle synthesis (Jagadeesh et al., 2016), nanoparticles synthesized for this study had nearly nanoscale morphology.

The nanoparticles synthesis was preliminarily confirmed through an UV-visible spectrometer. Brine shrimp and zebrafish embryo assays were conducted to analyze the cytotoxicity and embryonic toxicology of the nanoparticles respectively. As seen in a similar study, the nanoparticles showed relatively no toxicity in both the assays proving their safe nature and compatibility (Rajeshkumar et al., 2022). The toxicity levels were close to those seen in the control group where no intervention was added. If integrated accurately with the synthesized calcium oxide nanoparticles, this chemical aspect of the plant would enhance their properties significantly.



## 5. Conclusion

Green synthesized calcium oxide nanoparticles are proving to be a promising alternative to existing standard interventions with negligent toxicity levels. Subsequently the toxicity of the nanoparticles was analyzed through zebrafish embryos and brine shrimps in natural and suitable conditions. The tested green synthesized nanoparticles exhibited promising results in various concentrations in both the assays. The results indicate a bright prospect for these nanoparticles in the field of nanomedicine as both drug delivery agents and direct interventions with reduced toxicity and highly accurate activity. The biocompatibility of calcium oxide nanoparticles adds to their overall potency. Animal studies would be required to ensure the safety of these nanoparticles.

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### Disclosure Statement

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