

# Antibacterial effect of calcium carbonate nanoparticles on *Agrobacterium tumefaciens*

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## Abstract

**Aims:** Improvements in nanotechnology in the past decade has created various opportunities for the evaluation of biologic effects such as antibacterial effects of nanoparticles. The purpose of this study was to evaluate the antibacterial activity of calcium carbonate nanoparticles on two different bacteria including *Agrobacterium tumefaciens* and *Staphylococcus aureus*.

**Methods:** The antibacterial effect of calcium carbonate nanoparticles against mentioned bacteria was evaluated by dilution in agar containing medium and dilution in Broth medium. Each of prepared Broth media (10ml) was inoculated with 1 ml of bacterial suspension ( $10^6$  CFU/ml) and incubated. Sampling of culture media was performed in specific intervals and diluted as  $10^{-1}$ , to  $10^{-6}$ . Then 100 $\mu$ l of each sample was transferred to agar plates and was spread carefully and then incubated. Grown colonies were counted and MIC and MBC was determined.

**Results:** Calcium carbonate nanoparticles showed very good antibacterial effect and after 16 hours the bacteria were totally diminished. The lowest and highest MIC concentration of these nanoparticles in solid medium was 31.2 and 125 $\mu$ g/ml respectively. The MIC of calcium carbonate nanoparticles in Broth medium was two times more than the MIC concentration in solid medium, while different concentrations of ordinary calcium carbonate not only revealed antibacterial effects but also supported the bacterial growth.

**Conclusion:** Use of calcium carbonate nanoparticles as anti-microbial agent is recommended in different fields of food industry and agriculture and can be of importance considering health and economic issues.

**Keywords:** Calcium Carbonate Nanoparticles, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, Antibacterial

## Introduction

Today, one of the most important health priorities in medicine and agriculture is achieving a safe chemical disinfecting agent since the abundance of some bacteria in the environment has caused health problems. For example, *Agrobacterium tumefaciens* is a gram negative, aerobic, spore-free and soil borne bacterium [1]. Sometimes this bacterium may also be associated with the infections in humans and other animals [2, 3], but normally it is the cause of rim cancer or crown gall tumor in more than 90 economically important plant species, including seed plants such as apple, pear, apricot and other crops and ornamental plants such as Dahlia, Chrysanthemum and Rose [4]. Burr et al. introduced the crown gall of grape as the most important bacterial diseases of this plant throughout the world which is caused by *Agrobacterium tumefaciens* bio.var.3 [5]. The pathogen agent of this bacterium is a large tumor inducing plasmid (plasmid Ti) on which the pathogen genes are located [6, 7]. At the time of infection, a part

of this plasmid (T-DNA) is transferred to plant cells and enters its DNA [6, 7, 8, 9]. After presenting the T-DNA genes in the plant cells, the overproduction of auxins and cytotoxins result in the uncontrolled growth of the plant cells and eventually causes the tumor formation in the stem and root of the host plants [5, 6, 7, 8, 9, 10, 11]. This bacterium often enters the plant through the injured and damaged parts [9]. Burr et al. in 1984 showed that the pathogen was transferred through the grafts prepared from the grape tree and caused contamination and the disease symptoms occurred after a period of freezing or physical injury [12]. When an infected parent plant is used in order to prepare some seedlings and grafts, the pathogen agent can be spread across a broad region. Therefore, farmers are involved with numerous social and economic damages annually [5, 6, 13, 14]. In addition, *Agrobacterium tumefaciens* had also been reported in some human infections [15]. However, it was reported no evidence of this disease in humans. Also, *Staphylococcus aureus* spherical bacterium is aerobic and gram positive which is abundantly found in all

environments especially hospitals and creates numerous health problems. This avoids the necessity of continuing the disinfection. Therefore, finding a useful disinfectant substance to control these bacteria growth can prevent the losses. Therefore, different chemical and biological methods with bactericidal or inhibitory effects have been used to control contamination by these organisms [16]. Furthermore, improvements in nanotechnology and science in the past decade had created many opportunities for studying the biological effects such as nanoparticles antibacterial effects [17]. There are several studies emphasized the different physicochemical aspects of materials in the typical and nano sizes. The aim of this study was to evaluate the effect of antibacterial activity of calcium carbonate nanoparticles on the growth of *Agrobacterium tumefaciens* as a gram negative bacterium and *Staphylococcus aureus* as a gram positive bacterium.

## Methods

**Isolation of bacteria:** In this experimental study, the plant samples with crown gall tumor were collected from the farms and gardens of the different regions of Iran and some parts of the grafts and roots were cut by sterile scissors and were transferred to the laboratory in nylon bags or plastic containers. The preparation of samples was done by the recommended method of Moore et al. [4]. This method is briefly as follows: first the surface of the obtained plant samples was washed well with water and dark and necrotic parts of tissue were removed with a sterile scalpel and were washed with the detergent with the concentration of 2% for a few minutes. Then the samples were washed with sterile distilled water and the parts with suspected tumor were separated by sterile surgical blade. 2 to 3 pieces from different parts of gall were put in a sterile Petri dish. Then the collected pieces were crushed in a sterile porcelain mortar after chopping. The crushed tissue was transferred to the test tubes containing sterile distilled water. The tubes containing the sample were vortex and cultured with sterile loop from suspensions prepared from the tumors on PDA+CaCo<sub>3</sub> (0.5% w/v) and Yeast Mannitol Agar in lines and incubated at 28°C for 48 to 72 hours. After incubation, the grown colonies were investigated from macroscopic view. The round colonies with smooth surface and mucoid condensation with pearl white to brown-cream color without the transparent halo caused by acid formation in PDA+CaCo<sub>3</sub> (0.5% w/v) medium were selected. To access pure cultures, each

colony was separately cultured several times in PDA+CaCo<sub>3</sub> (0.5% w/v). To identify the isolated bacteria, all obtained colonies were coded and identity tests including microscopic, biochemical and molecular identification tests were conducted.

**Biochemical studies to determine the identity of isolates:** Biochemical tests including oxidase, catalase, indole production, lactose glucose oxidation and its conversion to 3-ketolactose, salt tolerance of 2% in nutrient Glucose Agar medium (NGA), the use or non-use of malonate and citrate, the induced changes in Litmus Milk, Pellicle formation (a brownish appendix on the surface) in ferric ammonium citrate, production or no-production of acid in PDA+CaCo<sub>3</sub> (0.5% w/v) were conducted. The ability to grow at 35°C was also studied.

**Evaluation of the antimicrobial activity:** In vitro condition, the antimicrobial activity of calcium carbonate nanoparticles on the isolated *Agrobacterium*, *Agrobacterium tumefaciens* LB4404 and also *Staphylococcus aureus* were evaluated by two methods of the dilution in agar containing medium and dilution in Broth medium.

Evaluation of the antimicrobial effect of calcium carbonate nanoparticles with the method of dilution in agar containing medium:

The characteristics of calcium carbonate nanoparticles powders with the standard Code of NNS12ca were included:

- Dry specific gravity (g/cm<sup>3</sup>): 2.5-2.4
- The mean of the particle size (nm) ≤ 50

The susceptibility of *Agrobacterium tumefaciens* to the calcium carbonate nanoparticles was studied using the standard method of the serial dilution preparation in agar containing medium [18, 19]. Double lessening dilutions of the calcium carbonate nanoparticles with the initial concentration of 0.1% (1000µg/mg) in TSA (Tryptic Soy Agar) were prepared. Thus, the obtained concentrations included 500, 250, 125, 62.5, 31.2, 15.6 and 7.8µg/mg in the TSA. Then, 50 to 100ml of the 24-hour cultures of *Agrobacterium* which were set based on the 0.5 turbidity of McFarland standard was transferred to the plates containing the certain concentrations of calcium carbonate nanoparticles, laboratory calcium carbonate (Merck Company; Germany) and the controlled plates which did not contain the calcium carbonate and were distributed in the medium with a glass sterile applicator. Then, the inoculated media were incubated in 28°C for 48 to 72 hours. The cultivated medium with the slightest concentration of calcium carbonate nanoparticles in which the number of colony less than the inoculated sample was observed and no growth was observed in

the higher concentrations was considered as the least inhibitory concentration from the growth of bacterium (MIC).

#### Antibacterial effect of calcium carbonate nanoparticles with the method of broth dilution:

The antimicrobial effect of calcium carbonate nanoparticles was studied using the standard method of the successive dilutions preparation in TSB medium (Tryptic Soy Broth) [20].

In this method, the double lessening concentrations were prepared from nano calcium carbonate and normal calcium carbonate. In the case that, 1ml of the initial concentration 0.1% of nano calcium carbonate and normal calcium carbonate was separately added to each of the first series of eight test tubes containing 1ml of TSB sterile medium, and the consecutive concentration (as in the previous method) were obtained. Finally, 1mm was discarded from the last tube. 1ml bacterial suspension containing  $10^6$ CFU/ml was added to each tube containing the different concentrations of calcium carbonate and incubated at  $28^\circ\text{C}$  for 48 hours. During the incubation in times of 0, 4, 8, 12, 16, 20 and 24 hours, the dilutions of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were prepared by removing a sample consisted of 100ml of each tube containing different concentrations of calcium carbonate and also the control tubes (1ml suspension  $10^6$ CFU/ml) and by using the TSB sterile broth medium and by respecting the aseptically condition. Then, 100 $\mu\text{l}$  from each of the prepared dilutions was transferred to the solid medium and was completely spread by a glass applicator. The inoculated media at  $28^\circ\text{C}$  were incubated for 48 hours. Then, by counting the number of the grown colonies, the effect of each

calcium carbonate concentration was studied. In the case that the growth of bacteria based on the turbidity in Broth media was evaluated. Then, 0.1ml was transferred to the agar containing media from each of the media in the intervals of 0, 2, 4, 12, 16, 20, 24, 36, and 48 hours, and was spread well and incubated at  $28^\circ\text{C}$  for 48 hours. After incubation, the number of the grown colonies was counted. The minimum concentration inhibiting the growth of *Agrobacterium* (MIC) was considered the concentration from calcium carbonate nanoparticles which created a number of colonies equal to or less than the number of inoculated organism in the transparent tubes. The minimum lethal bacterial concentration (MBC) was the first transparent tube after the MIC tube in which had no colony growth.

The standard and well-known *Staphylococcus aureus* ATCC6538 was used as control. This organism was cultured by the methods of several-parts culture in a plate and also the linear culture to the length of 2cm in the agar nutrient cultivated medium containing 62 $\mu\text{g}/\text{mg}$  of the nano calcium carbonate and was incubated for 24 hours at  $37^\circ\text{C}$ .

## Results

In the semi-specific medium, the colonies had grown with the appearance of mucoid, pearl white to cream color. All colonies were gram negative with the polymorph formation. Biochemical bacteria adapted with the *Agrobacterium* species were selected and encoded. The characteristics of the isolated bacterial species were presented in Table 1.

**Table 1-** Biochemical characteristics of *Agrobacterium tumefaciens* isolates

Tests→ Bacteria↓	Aerobic	Oxidase	Cotolactoz-3	Citrate Ferric Ammonium	Acid in PDA-CaCo3	Salt 2%	Meso- Eritrol	Mannitol	Citrate
<i>Agrobacterium tumefaciens</i> 1	+	+	+	+	-	+	-	+	-
<i>Agrobacterium tumefaciens</i> 2	+	+	+	+	-	+	-	+	-
<i>Agrobacterium tumefaciens</i> 3	+	+	+	+	-	+	-	+	-
<i>Agrobacterium tumefaciens</i> 4	+	+	+	+	-	+	-	+	-
<i>Agrobacterium tumefaciens</i> 5	+	+	+	+	-	+	-	+	-

The suspension of 18-hour used bacterial culture had the initial concentration of  $5 \times 10^6$ CFU/ml.

The results of investigating the antibacterial effect of different calcium carbonate nanoparticles and determining the MIC and MBC for each of 5 strains of the isolated *Agrobacterium tumefaciens*, *Staphylococcus aureus* ATCC6538, and *Agrobacterium tumefaciens* LB4404 using the dilution in agar containing medium and dilution in Broth

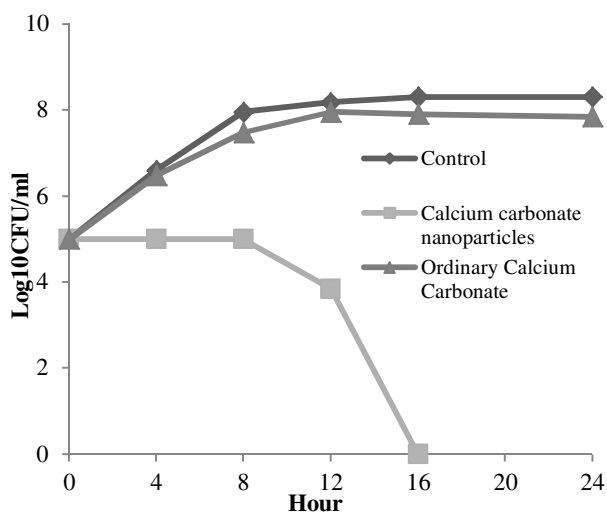
medium were shown in Table 2. The MIC of the calcium carbonate nanoparticles for the strains of *Agrobacterium tumefaciens* biovar. 1 (No. 1) and *Agrobacterium tumefaciens* biovar. 1 (No. 4) and also for the strain of *Agrobacterium tumefaciens* LB4404 was double of its amount in the Broth medium compared to the solid medium. Similarly, the MBC of the calcium carbonate nanoparticles in the Broth medium in some strains was two times more than the

MBC concentration of the same material in solid medium.

**Table 2-** The amount of MIC and MBC ( $\mu\text{g/ml}$ ) calcium carbonate nanoparticles on microorganisms

Methods of dilution preparation→ Bacteria↓	In agar containing medium		In Broth medium	
	MIC	MBC	MIC	MBC
<i>Agrobacterium tumefaciens</i> Biovar 1 (no. 1)	31.2	62.5	62.5	125
<i>Agrobacterium tumefaciens</i> Biovar 1 (no. 2)	31.2	62.5	31.2	62.5
<i>Agrobacterium tumefaciens</i> Biovar 1 (no. 3)	31.2	62.5	31.2	62.5
<i>Agrobacterium tumefaciens</i> Biovar 1 (no. 4)	62.5	125	125	250
<i>Agrobacterium tumefaciens</i> Biovar 1 (no. 5)	31.2	62.5	31.2	62.5
<i>Agrobacterium tumefaciens</i> (LB4404)	31.2	62.5	62.5	125
<i>Staphylococcus aureus</i>	125	250	125	250
The average mean	44.6	98.2	67	134

However, in both methods, the concentrations of 31.2-250 $\mu\text{g/ml}$  calcium carbonate nanoparticles showed growth preventing effect on all tested bacteria. While all the grown bacteria in the cultivated solid and Broth media containing the normal calcium carbonate had grown well with the concentrations of 0.1%, 0.5%, and 1% and the cream-white colonies with a diameter of 2-3mm were developed. Similarly, no growth was observed in the standard strains of *Staphylococcus aureus* ATCC6538 (as a control) after 24 hours of the incubation in 37°C.



**Diagram 1-** The growth of *Agrobacterium tumefaciens* was compared in the medium containing nano-calcium carbonate (250 $\mu\text{g/ml}$ ) and the medium containing the normal calcium carbonate (5mg/ml) and the control medium (calcium carbonate-free).

Calcium carbonate nanoparticles with the concentration of 250 $\mu\text{g/ml}$  completely stopped the growth of *Agrobacterium tumefaciens* after 16 hours. The fashion of the growth and reproduction of the organisms had no effect on the growth of bacteria in the control medium and the medium containing 5mg/ml the normal calcium carbonate, while the initial count of bacteria in the medium containing the concentration of 250 $\mu\text{g/ml}$  of calcium carbonate nanoparticles had decreased with time and reached to zero after 16 hours (Diagram 1).

## Discussion

In order to isolate and identify the different species of *Agrobacterium*, a high concentration of calcium carbonate was used in the culture medium, because 0.5 to 2% of calcium carbonate concentration was used for identification and maintenance of these bacteria [21, 22] which was not affordable in terms of the quantity. The nanotechnology can change the quantity-qualitative characteristics of the material in the forms of nanoparticle, and this technology can also create a new form of the material which shows a different effect of the concentration of the same material in the weight range (g) in the nanogram concentration [23]. Therefore, calcium carbonate nanoparticles were used in this study, because it was thought that using the calcium carbonate nanoparticles in bacterial culture medium was economically affordable. It was wonderfully and randomly observed that the forms of calcium carbonate nanoparticles not only did not result in enhancing the growth of *Agrobacterium tumefaciens* but also they prevented the intensity of its growth. In fact, in this study, the effect of nano calcium carbonate and the laboratory calcium carbonate were studied on the growth of a group of gram negative bacteria and one positive-gram bacterium. The results of this study showed that nano calcium carbonate had the antibacterial effect on the tested bacteria in very low concentrations, whereas the same bacteria had grown well in the medium containing the normal calcium carbonate. In other word, the normal calcium carbonate had shown no antibacterial effect in contrast to the antibacterial effect of nano calcium carbonate. Thus, the antimicrobial potential of calcium carbonate nanoparticles was determined as a plant pathogen by measuring MIC and MBC of *Agrobacterium*. The notable finding in this study was that the antibacterial effect of calcium carbonate nanoparticles in the solid medium was more than the effect of this substance in the Broth medium. As it was shown in Table 2, the

MIC concentration in the Broth medium had decreased two times for both the gram negative and positive-gram bacteria. In addition, the obtained results showed that the MIC of calcium carbonate nanoparticles for the gram negative bacteria was lower than the MIC of this substance for the positive-gram bacteria. The reason why the MIC concentration of calcium carbonate nanoparticles in a solid medium was the half of the MIC concentration of this substance in the Broth medium was unclear. But the significant point is that other researchers had shown that the metal nanoparticles show better antibacterial effect in the solid media which its cause was considered the combination of the nanoparticle and the available polymers in the medium and formation of composite (24).

Moreover, during recent years, similar antimicrobial effects have been reported in solid forms such as ceramics using the effects of the optical radiation (photochemical) resulting from the titanium oxide nanoparticle compounds [25]. However, the photochemical effects were not investigated in this study. Regarding the importance of this issue, further studies are recommended on both antimicrobial effects of calcium carbonate nanoparticles in solid medium and photochemical effects.

However, during recent years, the antibacterial effects of various nano materials such as nanoparticles of silver, copper, and titanium in different forms have been reported [26, 27], but regarding the study on available resources, no documentary evidence has been reported on the antimicrobial use of calcium carbonate nanoparticles and this study was the first report on this subject in Iran and the world.

Since the preparation of calcium carbonate nanoparticles is affordable compared to metal nanoparticles, the study on the application of the antibacterial effects of this material in food, agriculture and health and medicine can be a pathfinder to control the bacterial pollution.

## Conclusion

Calcium carbonate nanoparticles have antimicrobial effects. The lowest and highest MIC concentration against *Agrobacterium tumefaciens* gram negative bacteria in solid medium were 31.2 and 62.5 µg/ml, respectively. Similarly, the MIC against *Staphylococcus aureus* was two times more than the MIC against gram negative bacteria. In all cases, the MBC of calcium carbonate nanoparticles was two times more than the MIC. The significant point is that the MIC of calcium carbonate nanoparticles in Broth

medium is two times more than the MIC in solid medium. While the different concentrations of the normal calcium carbonate not only have antibacterial effects but also enhance their growth. Considering the above findings, calcium carbonate nanoparticles are suggested as a candidate for production of antimicrobial drugs used in various fields of medicine, food industry and agriculture.

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