DAVAO RESEARCH JOURNAL

https://davaoresearchjournal.ph P-ISSN 2244-4432 E-ISSN 2984-7125

# Biosynthesized calcium oxide from calcined conch shells: A potential bactericidal agent against *Escherichia coli*

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Submitted: 26 Jun 2024
Revised: 03 Jul 2024
Accepted: 18 Jul 2024
Published: 13 Sep 2024

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OPEN ACCESS

## ABSTRACT

The occurrence of ineffective antibiotics and an abundance of waste shell has provided a research opportunity to study calcium compounds through natural recycling, derived from the abundant gleaned species of gastropod conch (*Canarium urceus*) shells. Particularly, calcium oxide (CaO) from waste shells were investigated as a value-added application for inhibiting pathogenic *Escherichia coli*. Hence, this study investigated its bactericidal activity through the Kirby-Bauer disc diffusion method to measure its growth inhibition ability. Waste conch shells were collected from Maitum, Barangay Dahican, City of Mati, Davao Oriental. Conch shell powder was obtained through calcination in a muffle furnace for two hours at 800°C ($CS_{800}$) and 900°C ($CS_{900}$), both at 50% and 75% (w/v) concentrations, for comparison. Among the four tested samples with three replicates each, results indicated that $CS_{800}$ (50%) exhibited the highest inhibitory effects against *E. coli*. Findings also revealed that samples at 50% concentration demonstrated higher antibacterial activity than those at 75%. This statistically suggests that concentration level significantly influenced the bactericidal activity, whereas the 100°C difference in calcination temperature did not. The antibacterial activity of calcined conch shells can be attributed to CaO through two mechanisms: primarily, the strong alkalinity of the medium, and secondarily, the generation of $Ca^{2+}$ and reactive oxygen species (ROS) on the surface of CaO, which cause cell wall rupture. Another possible mechanism is also associated with generating superoxides.

*Keywords:* Bactericidal, calcination, conch shells, *Escherichia coli*

**How to cite:** Ibañez, K. L. C., and Dedal, A. D. (2024). Biosynthesized calcium oxide from calcined conch shells: A potential bactericidal agent against *Escherichia coli*. *Davao Research Journal* (DRJ), 15(3), 33-44. https://doi.org/10.59120/drj.v15i3.228

## INTRODUCTION

*Escherichia coli* is a gram-negative, facultative anaerobic bacterium. While many people perceive this as harmless coliform bacteria, strains like Shiga toxin-producing *E. coli* are found to be causative organisms for various diarrheal diseases in humans (Mueller, 2023). According to the Centers for Disease Control and Prevention (2022), these microorganisms are commonly acquired through contaminated food or water. It can promote infections depending on the array of virulence-associated genes that they harbor. As a result, bacterial infection and subsequent inadequate disinfection pose daily concerns around the globe.

The most conventional approach to fighting resistant *E. coli* infections is using antibiotics, which are medications that destroy or impede the growth of bacteria. However, the World Health Organization (2023) addressed the emergence of dangerously rising antimicrobial resistance due to people's excessive and inappropriate use of these medical drugs, resulting in the ineffectiveness of many currently available medications. Notably, 1 in 5 cases of urinary tract infections caused by *E. coli* exhibited declined susceptibility to standard antibiotics, such as ampicillin, co-trimoxazole, and fluoroquinolones. This threatens our ability to treat common infections, including foodborne diseases. Hence, there is an increasing demand to develop new antimicrobial agents that reduce the use of antibiotics. This issue has directed researchers to isolate and identify potential bioactive chemicals from other resources.

At present, waste shells are utilized as a bioresource of calcium carbonate in catalyzing calcination to produce calcium oxide (Zhang et al., 2018). Hence, conch shell's ability to produce a chemically synthesized product—calcium oxide (CaO)—may also hold a strong and broad-spectrum of antimicrobial activity against bacteria, fungi, and viruses (Tsukuda et al., 2021). Experiments revealed that bio-calcium oxide derived from heated scallop shells (Gultekin and Kucukates, 2019; Omura et al., 2024) and oyster shells (Tongwanichniyom et al., 2021; Tran et al., 2023) are found to be effective antibacterial disinfectants. These shell species from Turkey, Japan, Thailand, and Taiwan exhibited various lengths of inhibition on tested bacteria strains. This implies that habitat location and shell type determine their specific chemical composition, suggesting that the accumulation of new findings from other shell species should also be examined. This will provide new information for further experimentation to achieve optimal modifications. Accordingly, the bactericidal activity of the understudied *Canarium urceus* conch shells against *E. coli* has to be further explored.

In the Philippines, a study by Cadano et al. (2021) recorded that the country produces more than 250,000 metric tons of seafood waste annually. Through effective shell utilization, severe soil and water contamination and pollution can be eradicated. Coincidentally, a catch assessment of commercially essential gastropods from Guang-Guang, Mati, Davao Oriental showed that the most abundant species caught by gleaners is the *Canarium urceus* little bear conch (Maynawang and Macusi, 2023). This data suggests that conch shells contribute mainly to the piled-up waste products in the area.

Aside from its promising pharmaceutical application, this paper also aimed to draw attention to the experimental use of conch shell wastes to mitigate its environmental burden. Therefore, this study hypothesized that calcined conch shells exhibit bactericidal activity, mediated through the release of CaO and subsequent antibacterial mechanisms. This experiment can significantly offer new insights into its practical application as effective bactericidal agents in medical settings while addressing the global emergence of antimicrobial resistance.


DOI: https://doi.org/10.59120/drj.v15i3.228

## MATERIALS AND METHODS

### Study area

Conch shell samples were collected at Maitum, Guang-Guang, Barangay Dahican, in Mati, Davao Oriental (Figure 1). This is a residential area located near a coastline. Equally important, the site is known to be a favorable habitat for abundant marine organisms, specifically mollusks (Bersaldo et al., 2021; Macusi and Tipudan 2020). Hence, coastal communities could glean different species from which adjacent communities could benefit. Marine gastropods are one of the area's primary foods sold and consumed by residents. Notably, a catch assessment of little bear conch species or Aninikad showed that the area was highly exploited by gleaners for gastropods (Maynawang and Macusi, 2023). With growing population found in surrounding communities, this resulted to increased shellfish consumption, which meant more accumulation of seashell wastes dumped by the coastal communities. This was the imperative of the study, to initiate other productive use of waste shells. The assessment of its antibacterial activity took place at the Davao Oriental State University, specifically in the Microbiology Laboratory.

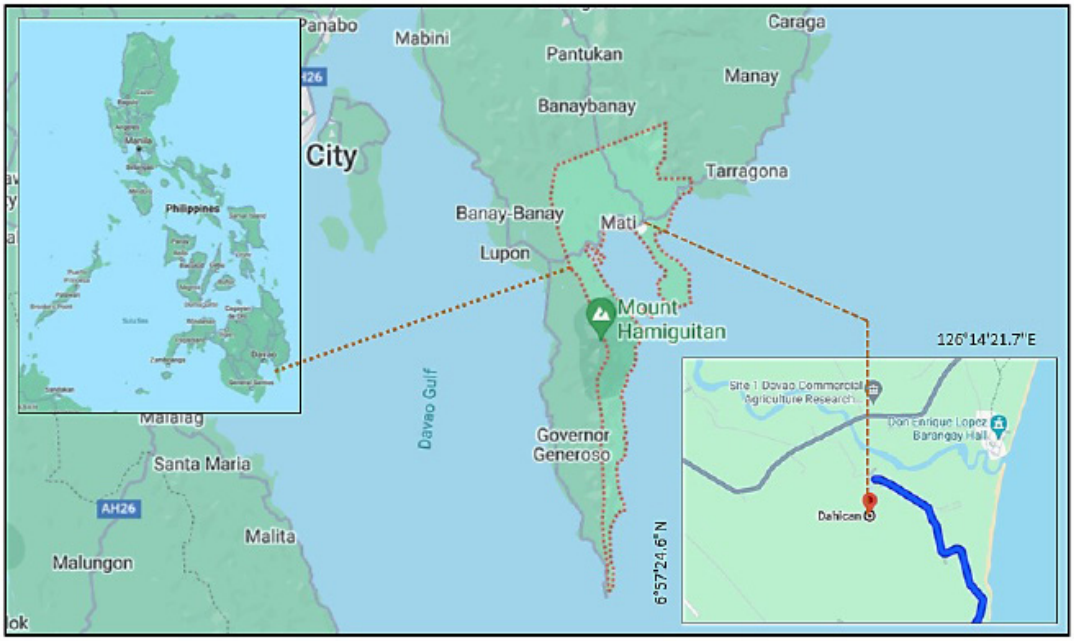


**Figure 1.** The study site located in Maitum and Davao Oriental State University in Barangay Dahican (lower right), City of Mati (orange outline), Davao Oriental, Philippines (upper left).

### Collection of sample and removal of background microbiota

Among the Strombidae gastropod family species, the shells of little bear conch (*Canarium urceus*), locally known as *Aninikad*, were the target species used. A total of 250 waste conch shells were obtained from houses in the residential community of Maitum, Guang-Guang, Barangay Dahican, City of Mati, Davao Oriental. In pursuit of natural recycling, all seashells utilized in this study were waste products where the soft bodies were already consumed or discarded.

The preparation of samples was conducted following the procedures detailed by Srey et al. (2014), with subsequent modifications. After collection, conch shells were cleaned using a wet brush to completely rid soft bodies and scrape off observable dirt. Then, shells were placed inside a cloth and smashed with a hammer to break down fragments. Cleaned conch shell pieces were soaked in distilled water for an hour to remove residues. After rinsing, prepared shell pieces were further disinfected by soaking them in 70% ethanol at room temperature for another hour to ensure the removal of other microbiota

possibly present in the shell. Shells were properly dried in the laboratory oven set at 170˚C for an hour.

**Calcination of conch shell sample**

Prepared conch shell fragments were heated through calcination in a muffle furnace (Vulcan A-130 Furnace, USA). For comparison, this study utilized two different temperatures: (1) the standard temperature of 800˚C as recommended by Nordin et al. (2015) and (2) a higher temperature of 900˚C based on the study of Aydin and Kalemtas (2021). Both sample sets were subjected to heat for 2 hours in gradual increase to obtain proper calcination of seashells. It has been proven that when the shell is heated at 700°C or above, shell microparticles convert its calcium carbonate ($CaCO_3$) into calcium oxide (CaO) (Alvarado and Quintana, 2022; Dampang et al., 2021; Nordin et al., 2015; Tongwanichniyom et al., 2021). This calcination process is presented in the following equation:

$$CaCO_3(s) \rightarrow CaO(s) + CO_2(g)$$

After calcination, shell fragments were further pulverized using mortar and pestle. The resultant solid product was sifted using a sterilized stainless steel sieve to obtain 150µm-sized microparticles. Lastly, the powder was stored in air-tight wrapped crucibles inside a desiccator. Figure 2 presents the process of obtaining biosynthesized CaO based on Sadeghi et al. (2019) with the above mentioned modifications.

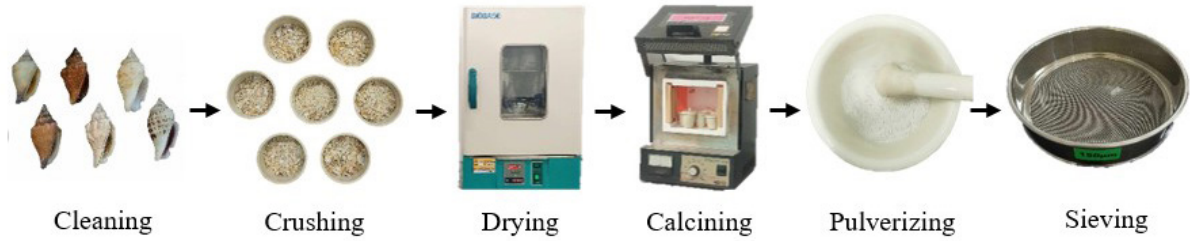


**Figure 2.** Process of calcium oxide production through calcination of conch shells.

**Culture medium preparation and inoculation of *Escherichia coli***

The bacterial strains were obtained from the verified *E.coli*-infected samples gathered by selected fourth-year research students of Bachelor of Science in Biology at Davao Oriental State University. Through subculturing, new cell cultures were made by transferring some cells from the previous culture to the fresh growth medium. Identified *E. coli* colonies gave a distinctive metallic green sheen (Aryal, 2022a).

This study used EMB agar as an efficient culture method (Bonnet et al., 2019, as cited in Basavaraju and Gunashree, 2023). In a beaker, 9 grams of the medium were suspended in 250 mL of distilled water. After carefully mixing, the desired concentration was heated to a boiling point using a magnetic stirrer and hot plate to dissolve the powder completely. Then, the agar mixture was sterilized by autoclaving at 121°C (250 °F) for 15 minutes. The mixture was dispensed into sterile petri dishes and cooled down for solidification. When the culture medium settled, the strain of *E. coli* was inoculated as soon as possible. Sterile cotton swabs were used to roll over an agar surface area and streaked for isolation. Then, the petri plates were incubated in an inverted position and kept protected from light at a temperature of 37°C. After 24 hours, a green metallic sheen formation was observed in the plates, and sterile cotton swabs and inoculating loops were used in carefully selecting the colonies of identified *E. coli* (Aryal, 2022a).

**Establishment of bactericidal activity**

For experimental treatments, two different CaO concentrations (50% and 75%) were prepared by dissolving calcined conch shell microparticles into 10 mL of


DOI: https://doi.org/10.59120/drj.v15i3.228

distilled water (Park et al., 2018) and stirred using a magnetic stirrer. The bactericidal activity of the treatments was determined using the paper disc diffusion assay technique. The standard medium used in this procedure is the Mueller-Hinton Agar or MHA (Clinical and Laboratory Standards Institute, 2019). The medium was prepared by suspending 28.5 grams of MHA in 1 liter of distilled water. Consequently, the mixture was stirred and heated to boiling, dissolving the medium completely. It was sterilized by autoclaving at 121°C (250 °F) for 15 minutes and cooled at room temperature. The mixture was poured into sterile petri dishes with uniform depth that was left to cool again (Aryal, 2022b). Subsequently, pure *E. coli* isolates were inoculated into the petri plates using sterilized cotton swabs. The entire surface was streaked equally by repeating the process two more times while rotating the plate approximately 60˚ each time (Tankeshwar, 2023).

A diameter of 6 mm Whatman filter no. 1 paper discs were obtained through a paper hole puncher. The discs were sterilized in a laboratory UV machine (Biobase, China) for 30 minutes. After sterilization, each paper disc was soaked in the four prepared sample sets of calcined conch shell microparticles and two control treatments (amoxicillin-clavulanic acid as positive control and distilled water as negative control). Then, impregnated discs were placed down in sterile petri dishes inoculated with *E. coli*. The plates were incubated at 37˚C for 24 hours. All experiments were performed in three replications. The bactericidal activities of all tests were measured using a caliper, measuring from the edges of the paper discs to the zone where bacterial growth was suppressed, also known as the zone of inhibition. All data were expressed in millimeters (mm).

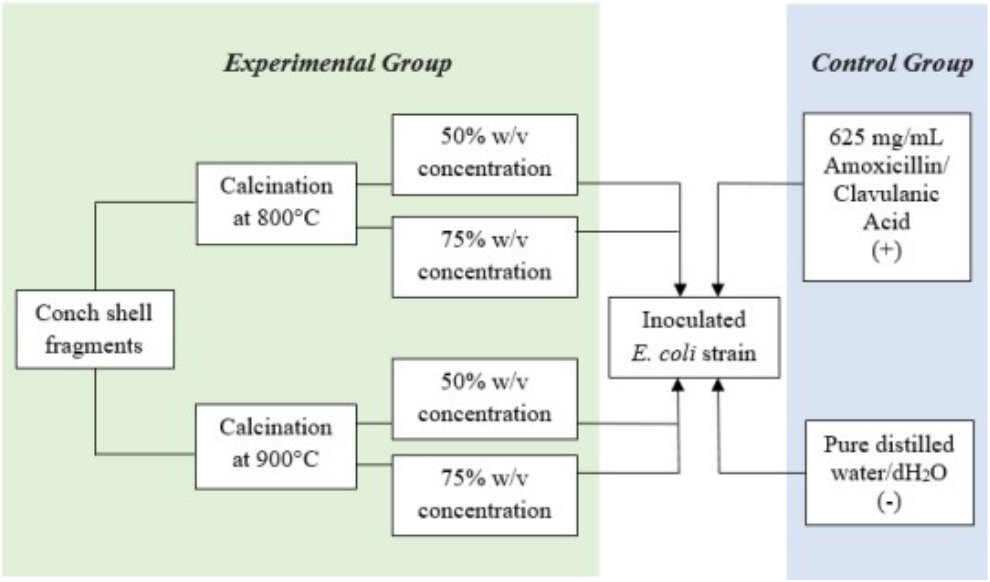


**Figure 3.** The schematic diagram of the bactericidal treatment comparison of the test compound and control group against inoculated *Escherichia coli*.

**Data analysis**

The zones of inhibition of each paper disc were used to record and calculate the bactericidal activities of all treatments. The findings of the experimental treatments were presented in a bar graph to compare the average bactericidal activities among various samples. To further analyze the gathered data, this study utilized a non-parametric test called the Mann-Whitney U Test. This method statistically determined the significant differences in the accumulation of inhibitory effects against *E. coli* among the different test concentrations and temperatures applied in the treatments.

## RESULTS

### Physical changes of conch shells

Shell fragments were originally a variety of brown and gray with hard, thick surfaces (Figure 4A). These turned into light brown after heating and drying at 170 °C (Figure 4B). After calcination at 800 °C ($CS_{800}$) and 900 °C ($CS_{900}$), the weight of shell fragments reduced by 5% and 10%, respectively, and became brittle. The color also changed to light gray and grayish white (Figure 4C and 4D).


**Figure 4.** Physical transformation of conch shells: Shells gathered and cleaned (A); Fragmented (B); Calcined ( C, D).

The changes in the physical characteristics and weight loss of conch shells were shown in Table 1. The sample calcined at a higher temperature had a higher weight reduction than those calcined at a lower temperature, following Boonyuen et al. (2015), as cited in Tongwanichniyom et al. (2021). Changes in the color and weight of the shells were caused by high-temperature thermal decomposition that induced oxidation reactions while releasing heat and gas.

**Table 1.** Physical characteristics and weight loss of heated conch shells, $CS_{800}$, and $CS_{900}$.

| Sample | Physical characteristics | | |
|---|---|---|---|
| | Color | Rigidity | Weight loss (%) |
| **Heated conch shell (170°C)** | Light brown | Hard | 0 |
| $CS_{800}$ | Light gray | Brittle | 4.95 |
| $CS_{900}$ | Grayish white | Brittle | 10.01 |

(Note: $CS_{800}$ = conch shells calcined at 800°C; $CS_{900}$ = conch shells calcined at 900°C.)

### Bactericidal activity of calcined conch shell microparticles

Findings of bactericidal activity (measured from the edge of the paper discs to the area of *E. coli* inhibition) were observed after 24 hours of incubation at 37˚C. The results of the study showed that calcined conch shell microparticles have antibacterial properties against *E. coli*, however, at various levels.

Among the four sets of experimental treatments, conch shell powder calcined at 800˚C (50% w/v) exhibited the highest bactericidal property with an average zone of inhibition (ZOI) of 3.3 mm (Figure 5A). This implies that the composition is the most effective in suppressing the growth of the bacteria among the four tested samples. Results also showed that 900˚C (50% w/v) has the second highest average with a ZOI of 3.0 mm (Figure 5B). It was also found that higher concentration levels (75% w/v) showed lower antibacterial efficacy. Conch shell microparticles calcined at 800˚C (75% w/v) had an average ZOI of 1.9 mm (Figure 5C), whereas 900˚C (75% w/v) had the lowest bactericidal property with an average ZOI of 1.7 mm (Figure 5D).


DOI: https://doi.org/10.59120/drj.v15i3.228

On the other hand, the control groups showed their standard results of inhibition zones. Figure 4E shows the bactericidal efficacy exhibited by the positive control with an average ZOI of 16.1 mm. Amoxicillin-clavulanic acid with a 625 mg antibacterial dosage displayed the largest bacterial inhibition area among all treatments. This implies that the antibiotic is significantly effective in stopping the growth of *E. coli.* In contrast, pure distilled water as a negative control showed no antibacterial activity against *E. coli* (Figure 4F), as no bactericidal properties, factors, or compounds were applied.

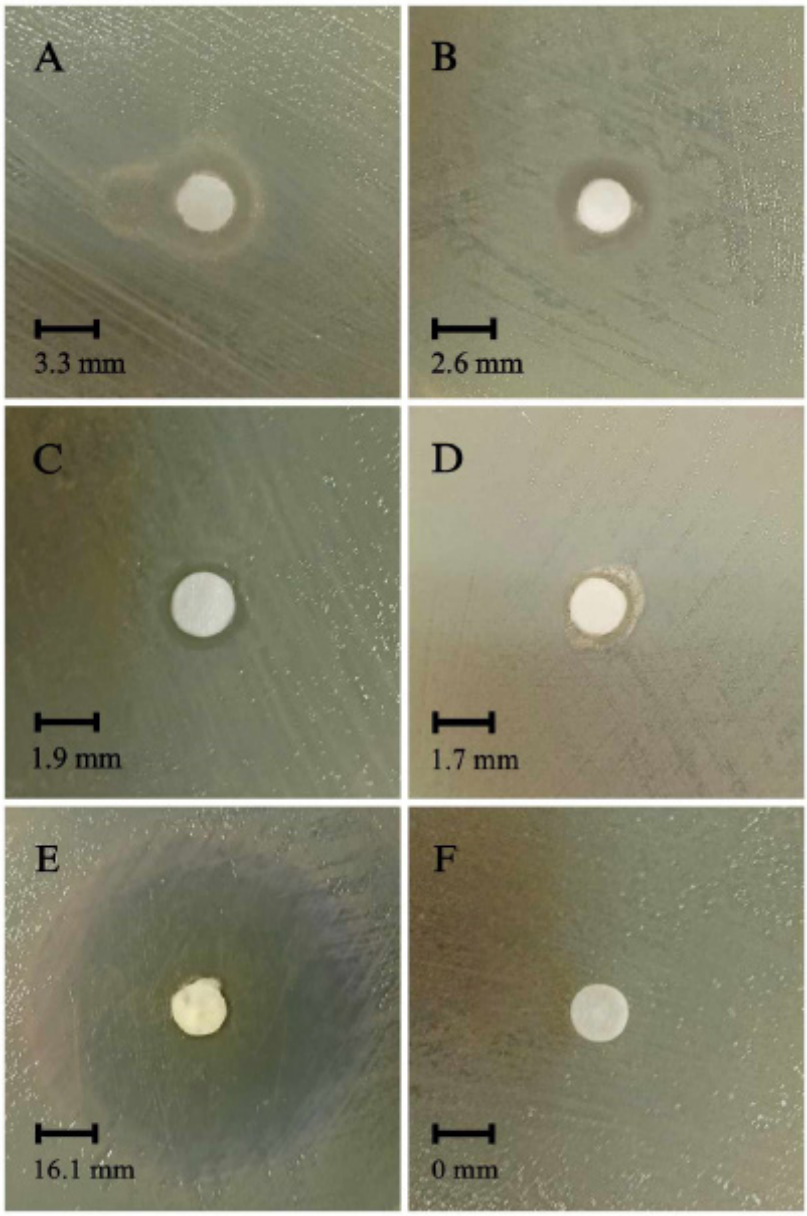


**Figure 5.** The average ZOI of different treatments: A) $CS_{800}$, 50%; B) $CS_{900,}$ 50%; C) $CS_{800}$, 75%; D) $CS_{900}$, 75%; E) positive control; and F) negative control.

**Significant differences in conch shell samples**

Figure 5 compares the mean ZOI among the four conch shell treatments tested in three trials. It can be deduced that samples with a concentration of 50% demonstrated higher bactericidal activity than those at 75%, regardless of the calcination temperature applied to the conch shell micro particles.

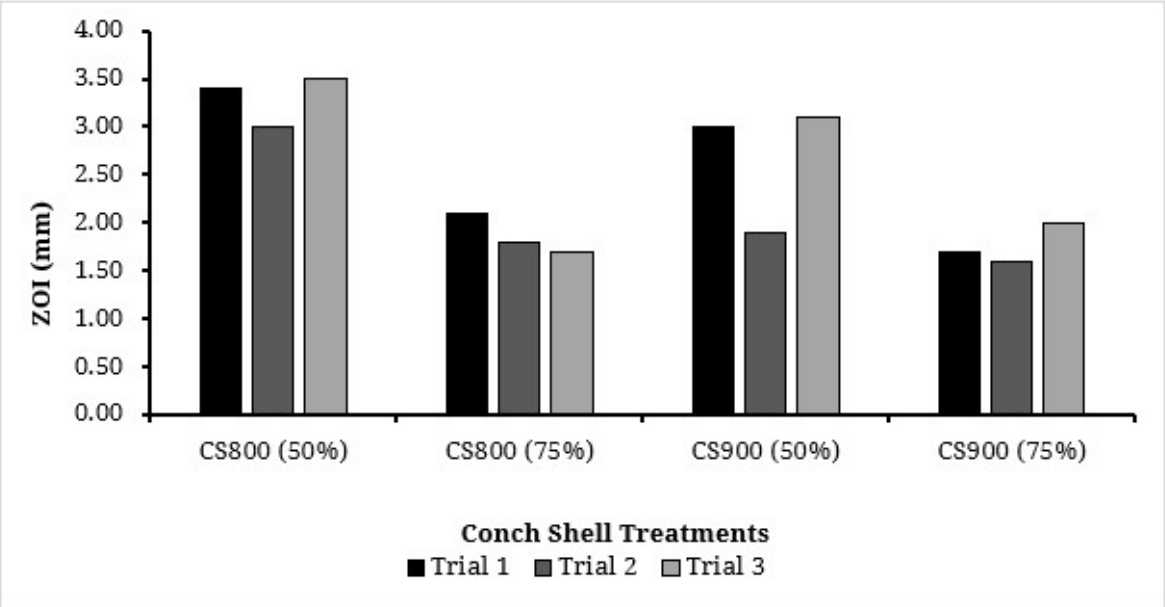


**Figure 6.** Mean zone of inhibition (ZOI) of calcined conch shell treatments against *Escherichia coli.*

**Statistical data analysis**

The Mann-Whitney U test was conducted to determine if there is a significant difference in the zone of inhibition (ZOI), indicative of bactericidal activity, between two concentrations of calcined conch shell microparticles—50% (w/v) and 75% (w/v). The null hypothesis claims that there is no significant difference between the concentration levels. This predicts that the distribution of ZOI is the same across concentration categories.

Microparticles at 50% (w/v) concentration had a significantly higher mean rank (26.44) in ZOI compared to the 75% (w/v) concentration (mean rank of 10.56). This suggests that the 50% concentration has a greater antibacterial efficacy. The Mann-Whitney U statistic was calculated to be 19.000, and both the asymptotic 2-tailed significance (*p*-value) and the exact significance were reported as 0.001. These *p*-values are substantially below the commonly accepted alpha level of 0.05 (Table 2).

**Table 2.** Mann-Whitney U test results for bactericidal activity of calcined conch shell by concentration level.

| Concentration | N | Rank average | Rank total | U test | *p*-value | Decision |
|---|---|---|---|---|---|---|
| 50% (w/v) | 18 | 26.44 | 476.00 | 19.000 | <0.001 | The null hypothesis is rejected. |
| 75% (w/v) | 18 | 10.56 | 190.00 | | | |

The Mann-Whitney U test was applied to assess whether there was a significant difference in the zones of inhibition (ZOI) of bactericidal activity at two different calcination temperatures of conch shell microparticles—800°C and 900°C (Table 3). The null hypothesis claims no significant difference between the temperature levels. This predicts that the distribution of ZOI is the same across temperature categories.

**Table 3.** Mann-Whitney U test results for bactericidal activity of calcined conch shell by temperature.

| Temperature | N | Rank average | Rank total | U test | *p*-value | Decision |
|---|---|---|---|---|---|---|
| 800°C | 18 | 20.00 | 360.00 | 135.00 | 0.391 | The null hypothesis is retained. |
| 900°C | 18 | 17.00 | 306.00 | | | |


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## DISCUSSION

### Bactericidal mechanism

Calcined conch shell microparticles dissolved in distilled water produced an aqueous solution of calcium hydroxide ($Ca(OH)_2$) with high alkalinity properties due to the addition of water, which could possess antibacterial properties (Dewi et al., 2019). This chemical reaction is due to calcium oxide being sensitive to hydrolysis, hence turning to $Ca(OH)_2$, as described in the equation below:

$$CaO\ (s) + H_2O\ (l) \leftrightarrow Ca(OH)_2\ (l)$$

The antibacterial activity of the tested compounds can be attributed to the calcium oxide and calcium hydroxide having high alkalinity, as well as having a strong reaction to the peptidoglycan of the bacterial membrane (Sadeghi et al., 2019; Tongwanichniyom et al., 2021). It can also be attributed to CaO by the generation of $Ca^{2+}$ and reactive oxygen species (ROS) on the surface of CaO, which also causes cell wall rupture. These factors may cause damage to the cell membranes of bacteria, hence the subsequent death of bacterial cells. Another possible mechanism for bactericidal activity of CaO is associated with generating superoxides (Sawai et al., 2001, as cited in Park et al., 2018). This mechanism is consistent with previous studies by Park et al. (2018), Sadeghi et al. (2019), and Tongwanichniyom et al. (2021), which reported that CaO solutions demonstrated antibacterial properties. Therefore, these findings indicate that the antibacterial activity was due to the generation of CaO.

The antibacterial activity of the treatments can be attributed to the strong alkalinity, generation of ROS, and strong reaction to the peptidoglycan of the bacterial membrane. This effect on the cell wall is also present in the bactericidal mechanism of the positive agent, Amoxicillin-Clavulanate, which is a combination of two different drugs: amoxicillin and clavulanic acid. Amoxicillin functions by attaching to penicillin-binding proteins, thereby blocking peptidoglycan synthesis, hindering cell wall formation, and ultimately causing bacterial destruction or lysis. Clavulanic acid, frequently paired with amoxicillin, also serves as a beta-lactamase inhibitor that extends its spectrum and counteracts resistance mechanisms, hence the strong antibacterial activity (Evans et al., 2023).

Furthermore, studies conducted by Park et al. (2018) and Tongwanichniyom et al. (2021) used relatively lower concentration levels than this study. This is a probable factor that may have affected the antibacterial activity of the samples, suggesting that concentrations 50% and 75% may be higher than needed and, hence, must be decreased. Moreover, producing calcium oxide through calcination can also cause a reversible reaction—reverting calcium oxide back to calcium carbonate when excessive carbon dioxide is absorbed. This factor can occur during paper disc soaking before bactericidal testing or when the sample is improperly handled.

Moreover, the use of calcined conch shells as a potential bactericidal agent also poses limitations in medical applications. Unlike other types of shells, conch shells have been less studied for this purpose. Therefore, to effectively apply calcined conch shell microparticles as a bactericidal agent, it is crucial to investigate how various processing and activation parameters impact their sterilizing effectiveness. An optimal modification must be analyzed to produce the best results.

### Statistical difference between concentrations

According to the Mann-Whitney U test results, the significantly low *p*-values lead to the rejection of the null hypothesis and to the conclusion that there is a statistically significant difference in the antibacterial effectiveness between the two tested concentrations of calcined conch shell microparticles. Therefore, the concentration of these microparticles significantly impacts their ZOI, with the

50% (w/v) concentration showing superior antibacterial activity compared to the 75% (w/v) concentration under the experimental conditions.

Different antibiotic concentrations can influence the efficacy of antimicrobials (Li et al., 2017). Thomas et al. (2012) stated that the activity of macrolides and aminoglycosides (i.e., antibiotics used against Gram-negative pathogens) are reduced in alkaline conditions; in this study, a high alkalinity environment was observed. Studies by Park et al. (2018) and Tongwanichniyom et al. (2021) also used relatively lower concentration levels to test the antibacterial activity of heated shells. This could be a probable factor that affected the bactericidal activity on the basis of its concentration level. This suggests that a higher calcined conch shell microparticle concentration does not necessarily correspond to increased bactericidal action. This analysis contradicts the study of Park et al. (2018), which stated that the bactericidal activity of CaO against biofilms increased with increasing concentrations of CaO. Therefore, there may be an optimal concentration for antibacterial purposes that should be considered when using calcined conch shell microparticles.

### Statistical difference between temperatures

Samples calcined at 800°C had a higher mean rank (20.00) compared to those calcined at 900°C (17.00), suggesting that the former had a generally larger ZOI. Despite the difference in mean ranks, the Mann-Whitney U test yielded a value of 135.00, with an asymptotic 2-tailed significance (*p*-value) of 0.391 and an exact significance of 0.406. Both *p*-values exceed the conventional threshold of 0.05 for statistical significance.

Based on the study by Nordin et al. (2015), a calcination temperature of 800˚C yields a higher amount of CaO than a calcination temperature of 700˚C and 900˚C. However, the results of this study suggest that both 800˚C and 900˚C temperatures have no significant differences in the bactericidal results of conch shells against *E. coli.* The findings of lower temperatures having higher antibacterial activity, however, align with the study of Desiate et al. (2019), which also compared two levels of calcination temperatures. It revealed that specific nanocomposite samples calcinated at lower temperatures were more active in killing *E. coli* bacteria than those calcined at higher temperatures.

Given that the *p*-values are greater than 0.05, it failed to reject the null hypothesis and concluded that there is no statistically significant difference in the median ZOI between the conch shell microparticles calcined at 800°C and those calcined at 900°C. Hence, this means that under the conditions tested, the calcination temperature does not significantly influence the antibacterial efficacy as measured by ZOI.

## CONCLUSION

Shells are utilized as a bioresource in producing calcium oxide that have shownpotential in a broad spectrum of antimicrobial activity against bacteria like *E. coli.* Thedangerous rise of antimicrobial resistance led to the initiative of this study investigatingthe bactericidal activity of *Canarium urceus* conch shells, which are understudied commonwaste products in the city. Among the experimented treatments, results suggested that conch shell calcined at 800˚C at 50% concentration is the most effective growth inhibitor susceptible against *E. coli.* This bactericidal result could have significant implications for medical and industrial applications, potentially reducing reliance on the declining antimicrobial agents and offering a sustainable alternative.

Further studies are necessary to fully understand and harness the bactericidal properties of conch shells to find optimal composition, but the initial findings suggest a promising avenue for future research and


DOI: https://doi.org/10.59120/drj.v15i3.228

development in antibacterial treatments. Therefore, this paper presents the following recommendations: Researchers may continue this study by adding modifications to the composition of the experimental treatment.Testing different concentration levels, preferably lower concentrations, is also suggested following experiments from similar literature. A similar study may be tested against other human pathogenic bacteria (e.g. *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella typhimurium*). Also, conch shells may be further examined and modified to determine their antibiofilm and preservation potential.

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 DOI: https://doi.org/10.59120/drj.v15i3.228