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# Effect of Zinc Oxide Nanoparticles and Calcium Ions on Thermal Stability of Peroxidase in *Calamintha officinalis* Moench

**Sorour Sepordeh Seydani**[1], **Fatemeh Shams Moattar** [2]✉

[1] Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[2] Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran

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✉ ***Correspondence to:***
Fatemeh Shams Moattar

**Email:**
fshams@ymail.com

**A B S T R A C T**

**Objective:** The antioxidant activity of plant extracts and essential oils is particularly important because of their beneficial physiological effects on cells and their potential for substitution by artificial antioxidants. One of the limitations in the use of active plant biomolecules in industry is their low thermal stability. To investigate the effect of zinc oxide nanoparticles (ZnO NPs) and calcium ions on enhancing the thermal stability of peroxidase enzyme (POD) from *Calamintha officinalis Moench* (COM), the present study was conducted.

**Methods:** In this study, after assessing the activity of peroxidase, the optimal pH and temperature of peroxidase were measured. The effect of nanoparticles on the thermal stability of the peroxidase enzyme was investigated at different temperatures and concentrations of 0.01 to 0.00001 mg/ml. Finally, the effect of calcium ions on the thermal stability of the desired enzyme was also measured at concentrations of 0.01 to 0.00001mg/mL. The control group was also considered to be the solution without $Ca^{2+}$ and ZnO NP.

**Results:** The optimal pH and temperature of the peroxidase enzyme were reported as 6.8 and 25 °C, respectively, with the highest thermal stability at 40 °C. There was no significant difference between the activity of the enzyme in different concentrations of zinc oxide nanoparticles. Based on our results, the activity of the desired enzyme in the presence of different concentrations of calcium showed that lower concentrations (0.00001mg/mL) led to an increase in enzyme activity in the first 1 hour.

**Conclusion:** According to our results, enzyme activity did not change at different concentrations of zinc oxide nanoparticles, but calcium ions may affect the thermal stability of peroxidase in the COM plant.

**Keywords:** Peroxidase, Thermal Stability, *Calamintha officinalis*, Zinc Oxide Nanoparticles, Calcium Ions

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## Introduction

When the ability of a biological system to eliminate or control free radicals is reduced, oxidative stress occurs, which is associated with the formation of reactive oxygen species (ROS) [1]. These compounds have a very destructive effect on cellular components and biological molecules, such as proteins, DNA, and lipids, and disrupt cell function [2, 3]. Numerous diseases, including atherosclerosis, hypertension, cardiac ischemia, diabetes, and cancer, are largely caused by oxidative stress [4, 5]. Some enzymes have been designed in living organisms to eliminate ROS, which include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)[6, 7].

Medicinal herbs' biologically active compounds and essential oils are frequently used to prevent and treat human illnesses [8]. The use of many synthetic antioxidants has side effects, and replacing them with plant extracts can be very helpful in this


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regard. Plant extracts have numerous beneficial biological effects on biochemical reactions occurring in human cells.

Plant peroxidases, one of the most important components of the antioxidant defence system, play a key role in the detoxification of ROS. Numerous uses for these enzymes can be found in the biotech, food, and pharmaceutical sectors [9, 10]. However, the thermal stability of these enzymes under industrial conditions remains a challenge [11]. Nanotechnology has opened a new horizon in biochemical research. Zinc oxide nanoparticles (ZnO NPs) have useful properties, such as antimicrobial activity, antioxidant effects, and the ability to modulate enzyme activity [12]. On the other hand, calcium ion ($Ca^{2+}$) as a second messenger in plant cells can affect the structure and function of many enzymes [13, 14].

Calaminta officinalis Muench (COM) belongs to the Lamiaceae family. This plant has a mint-like appearance and smell [15]. COM is one of the important medicinal plants, which contains bioactive compounds with strong medicinal properties [16, 17]. The antibacterial, antioxidant, and anti-inflammatory pharmacological activities of C. officinalis have also been mentioned in several studies [18]. Although the medicinal properties of this plant have been the subject of numerous studies, little is known about the effect of calcium ions and zinc oxide nanoparticles on its heat stability.

Stability is a key aspect in the design and introduction of drugs [19]. Understanding the mechanisms affecting plant enzyme stability may help develop novel techniques for maximizing plant enzyme activity in industrial settings. The findings of such research can be used to develop new plant-based medicines. To overcome limitations in enzyme stability under industrial conditions, recent studies have explored the use of nanomaterials and metal ions as stabilizing agents. Therefore, this study aimed to investigate the modulatory effects of zinc oxide nanoparticles and calcium ions on the thermal stability and catalytic activity of peroxidase in COM extract, with potential implications for industrial enzyme applications.

## Materials and Methods

### Plant collection and extraction

Initially, fresh leaves of the COM were collected from Tutaki village, the mountainous areas of Deylaman. The plant species was confirmed at the Gilan Agricultural and Natural Resources Research and Education Center, the place where the plant with herbarium code 6964 was deposited. The samples were ground after washing with distilled water and freezing in -70 °C. Extraction was performed using 0.5 g of leaf powder in 1 mL of potassium phosphate buffer (50 mM, pH 7.0). The homogenate was centrifuged at 14000 ×g for 4 min at 4 °C. The crude extract was obtained from the supernatant.

### Protein determination

The Bradford colorimetric method was used to determine the total protein concentration of the extract. This method is based on measuring the binding of Coomassie Brilliant Blue G-250 dye to protein molecules in comparison with known protein standards [20].

### Peroxidase Activity Assay

Peroxidase Activity Assay was performed using guaiacol as substrate in a reaction mixture containing ۹ mM guaiacol, ۴0 mM phosphate buffer (pH ۶/۸), ۲ mM $H_2O_2$,and 50 µL enzyme. Absorbance was monitored at 470 nm.

### Determining the optimal temperature /pH and thermal stability of the peroxidase

For the determination of the optimum temperature of enzyme activity, 150 mL of extract was mixed with 150 mL of guaiacol and $H_2O_2$ solution and incubated for 10 minutes in a water bath (25–50 °C). The absorbance was measured at 470 nm. The optimal pH for peroxidase activity was determined using 0.1 M phosphate buffer across a pH range of 5.8-8.0. Equal volumes of buffer and enzyme extract were mixed, and absorbance changes at ۴۷۰ nm were recorded every 30 seconds for 5 minutes at 25 °C. All assays were performed in triplicate. Finally, by plotting the absorption changes against different pH values, the optimal pH was obtained. The determination of thermal stability of peroxidase enzyme in the crude extract of Calaminta officinalis Muench was examined at temperatures of 30, 35, 40, 45, and 50 °C.

### Effect of ZnO NPs on the Thermal Stability of Peroxidase

In this part, the thermal stability of peroxidase in the crude plant extract was evaluated in the presence of zinc oxide nanoparticles using a spectrophotometric assay. In this study, nanoparticles with a size of 15–30 nm were purchased from Sigma-Aldrich. The results of the nanoparticle analysis of the samples by XRD show that the size of the zinc oxide nanoparticles was about 30 nm. XRD pattern and SEM micrograph of the purchased nanoparticles are shown in Figure 1.

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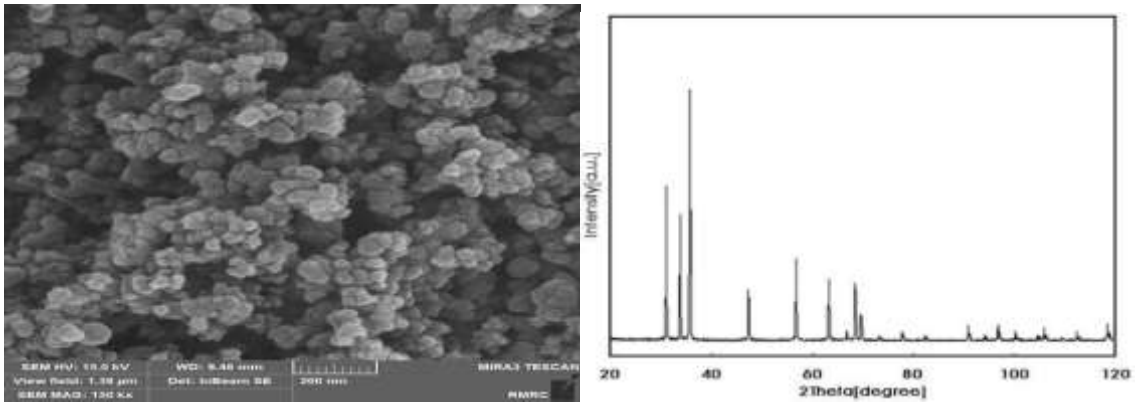



Figure 1. XRD pattern and SEM micrograph of the purchased nanoparticles

The enzyme extract was treated with varying concentrations of ZnO nanoparticles (0.01–0.00001 mg/mL), and its activity was measured. The solution without ZnO NP was also considered as the control group.

Effect of Calcium Ions on the Thermal Stability of Peroxidase

In the first step, 1% (w/v) calcium chloride solution was prepared and diluted to concentrations of 0.001, 0.0001, and 0.00001 mg/mL, then mixed with the enzyme extract for peroxidase activity measurement. Finally, enzyme extracts were incubated at room and refrigerated temperatures for 4 days, and activity was assessed every 24 hours. The solution without $Ca^{2+}$ was also considered as the control group.

## Results

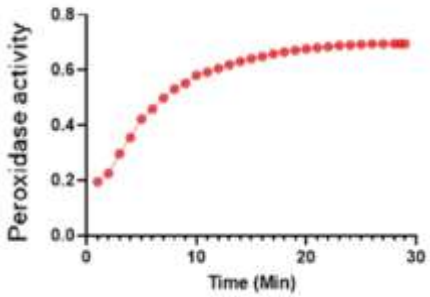
### Protein determination

The total protein concentration of the crude plant extract was measured by the Bradford method, and its standard curve was drawn using albumin. According to our results, the total protein concentration of the crude extract was 0.495 mg/mL.

### Peroxidase Activity Assay

Results of the activity of the peroxidase enzyme in crude extract at 25 °C are shown in Figure ۲. Based on our results, the activity of peroxidase increased until minute 26. The highest activity of the enzyme, 0.695 U/mg, was obtained. From minute 29, the enzyme activity reached a stable level.

Figure ۲. Activity of the peroxidase enzyme in the crude extract at 25°C




### Determining the optimal temperature /pH and thermal stability of the peroxidase

The enzyme activity was measured at temperatures of 5, 10, 15, 20, 25, 30, and 35 °C. The enzyme showed the highest activity at 25 °C (Figure ۳A). Activity of the enzyme was measured in

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the pH range 5 to 8. Results showed the highest activity at pH 6.8; therefore, this pH was considered the optimal pH for the peroxidase enzyme (Figure ۳B). The thermal stability of the enzyme was investigated at temperatures of 30, 35, 40, 45, and 50 °C. The enzyme remained completely stable at 40 °C, and its activity did not change after 50 minutes at this temperature (Figure ۳C).

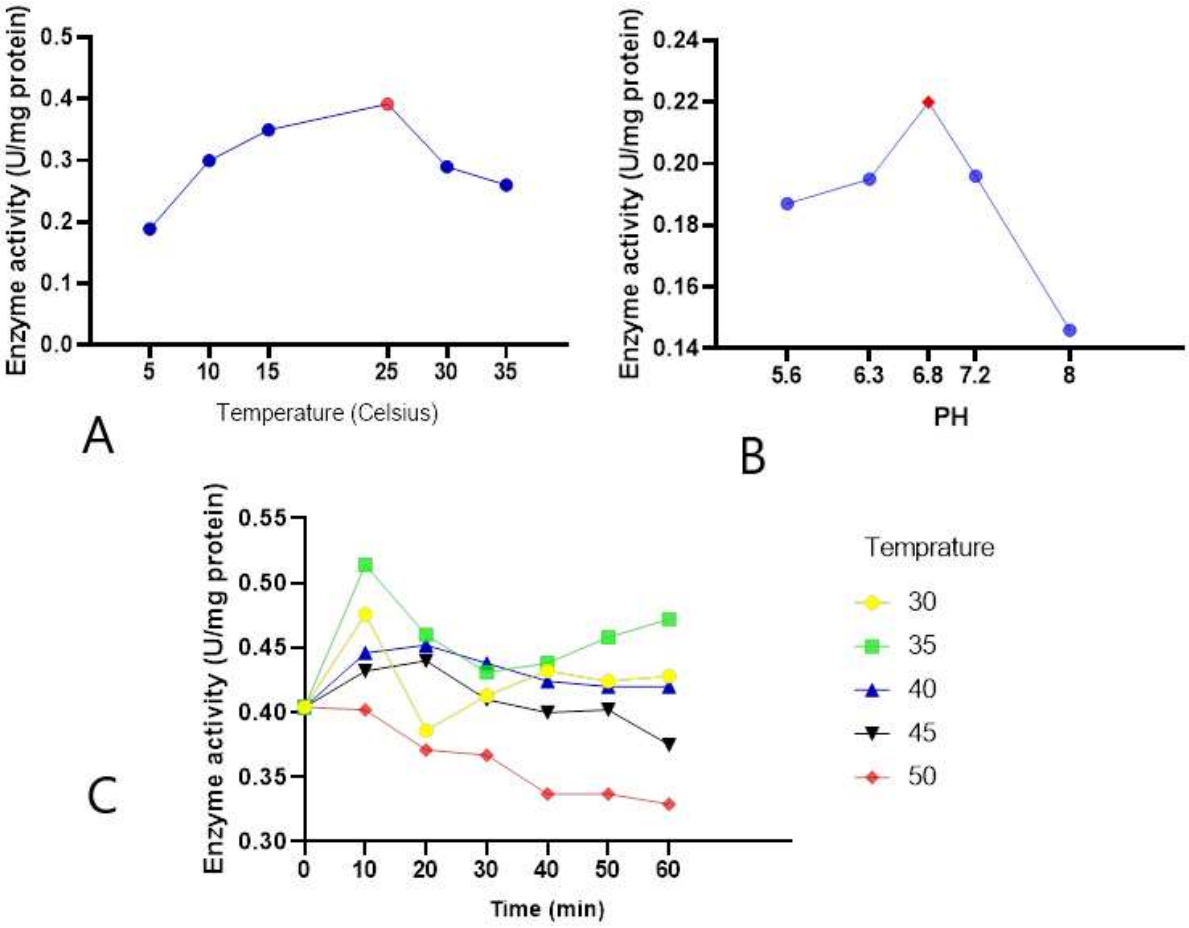



Figure ۳. Effect of temperature on peroxidase activity (A), effect of pH on peroxidase activity (B), and thermal stability of the peroxidase (C).

## Effect of ZnO NPs on the Thermal Stability of Peroxidase

The effect of different concentrations of ZnO NPs was investigated on the thermal stability of the peroxidase enzyme. According to the results, the highest peroxidase enzyme activity was reported at a concentration of 0.001 mg/mL. The lowest enzyme activity was reported at a concentration of 0.01 mg/mL, which is lower than the control value. In general, no significant changes in activity were observed at different concentrations of zinc oxide nanoparticles (Figure ۴).

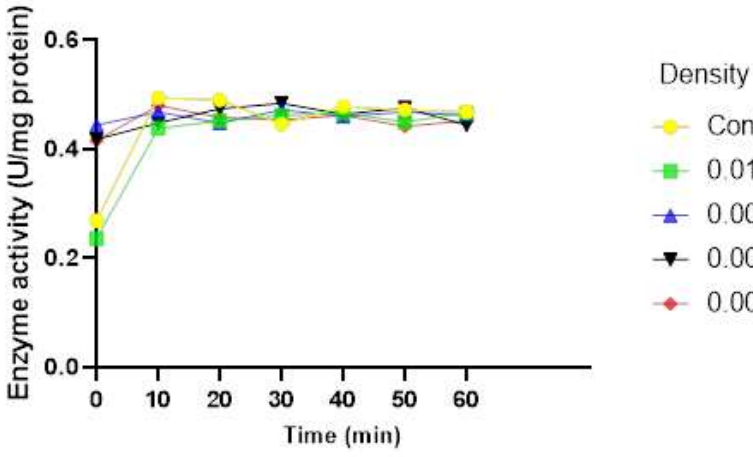


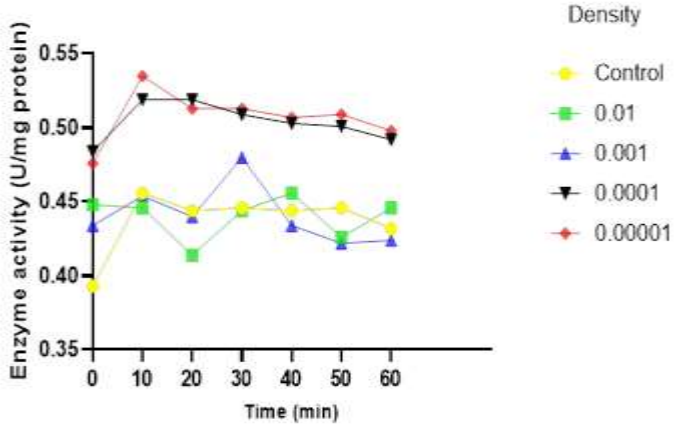

Figure ۴. Effect of ZnO NPs on the activity of peroxidase in thermal stability temperature

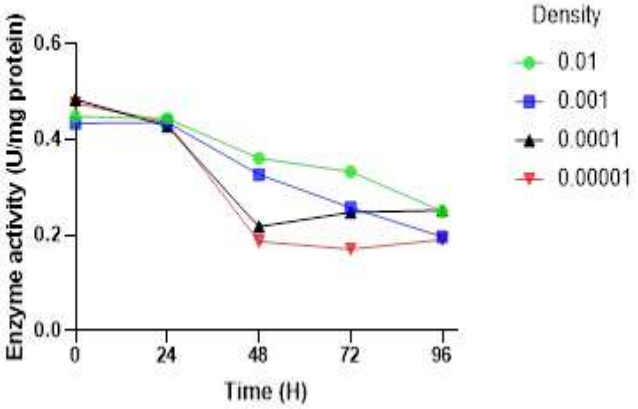
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## Effect of calcium ions on the Thermal Stability of Peroxidase

The activity of the peroxidase enzyme was investigated at different calcium concentrations at a stable temperature (40 °C). The highest enzyme activity was observed at a concentration of 0.00001 mg/mL. The results of this study showed that enzyme activity increased as the concentration decreased from 0.01 to 0.00001 mg/mL. (Figure ۵A). Examination of peroxidase enzyme activity in the presence of different concentrations of calcium at room temperature showed that the enzyme was stable at concentrations of 0.01 and 0.001 mg/mL during the first 24 hours. After 24 hours, a sharp decrease in stability was observed at all concentrations, which continued up to 48 hours (Figure ۵B). According to our findings on enzyme activity at refrigerated temperatures, a cold environment caused severe instability of enzyme activity in the presence of 0.01, 0.0001, and 0.00001 mg/mL calcium over different periods. Only at 0.001 between 24 and 72 hours, a relatively acceptable stability in enzyme activity was observed (Figure ۵C).



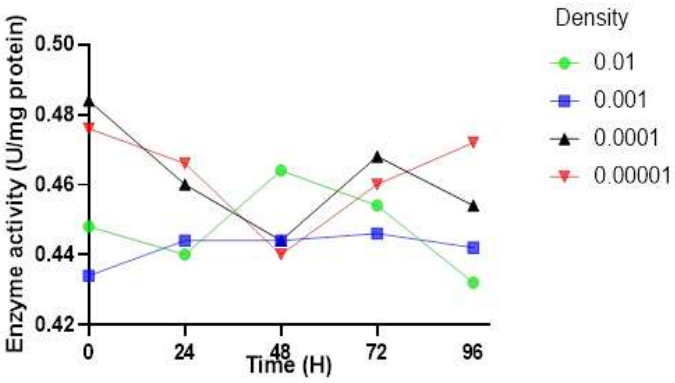




**Figure 5:** Effect of $Ca^{2+}$ on peroxidase activity at 40 °C (A), room temperature (B), and refrigerated conditions (C)

## Discussion

The antioxidant and free radical scavenging capabilities of natural compounds are among their most crucial roles in biological systems [21, 22]. Enzymes with peroxidase activity are widely found in microorganisms, plants, and animals. This class of catalysts utilizes hydrogen peroxide as an electron acceptor and performs biochemical reactions [22, 23]. Today, the application of peroxidase enzymes is significant in various fields, including environmental management, biotechnology, and pharmaceuticals [23, 24]. Therefore, the thermal stability characteristics of this enzyme are of particular importance for various users in industries. In this research, increasing the thermal stability of the peroxidase enzyme of the plant Calaminta officinalis Muench was targeted. In order to achieve this, enzyme activity was assessed while calcium ions and zinc oxide nanoparticles were present in varying concentrations.

According to the findings, the total protein concentration in the crude extract was 0.495 mg/mL, with the highest enzymatic activity of 0.695 U/mg, optimal temperature: 25 °C, optimal pH: 6.8, and highest thermal stability: 40 °C.

Our findings regarding the impact of zinc oxide nanoparticles on peroxidase's thermal stability demonstrated that the enzyme's specific activity at various zinc oxide nanoparticle concentrations did not differ significantly. Given the wide applications of ZnO nanoparticles in medical sciences, including wound healing, as antimicrobial, anti-inflammatory, antifungal, and anti-cancer agents, these nanoparticles were selected in our study. This type of nanoparticle with a large surface area can easily interact with enzymes. [25, 26]. Different

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results have been observed in relation to the effect of ZnO nanoparticles on enzyme activity. In a study conducted by Srivastava et al, to investigate the effect of zinc oxide nanoparticles on improving the thermal stability of enzymes, the Aspergillus cellulase enzyme was used. This enzyme was treated with zinc oxide nanoparticles to increase its thermal stability at 65 °C for 10 hours [۲۷]. Koupaei et al., also investigated the effect of zinc oxide nanoparticles on the stability of proteinase K after green synthesis. Based on their results, its thermal stability increases with increasing nanoparticle concentration [28]. In studies, the use of ZnO NPs does not always lead to an increase in thermal stability, and sometimes it does not even lead to a decrease in thermal stability at all. For example, in a study that examined the effect of zinc oxide nanoparticles on the stability and structure of egg white lysozyme, the results showed that increasing the concentration of zinc oxide nanoparticles was associated with a decrease in enzymatic activity and thermal stability of lysozyme [29]. In numerous studies, the impact of different nanomaterials on the thermal stability of enzymes has been documented. In a study by Cherian et al., cellulase immobilized on MnO2 nanoparticles showed thermal stability [30]. Zhang et al. conducted another study. Thermal stability was demonstrated by cellulase immobilized on functionalized magnetic nanoparticles. [31]. The different effects of nanoparticles are based on the type of enzyme, type of nanoparticle, temperature, incubation time, and concentration.

On the other hand, the effect of calcium ions on the activity of peroxidase at thermal stability temperature, room temperature, and refrigerated temperature was also investigated. Plant peroxidases contain two calcium ions, which are essential for the structural and thermal stability of the enzyme. In the absence of calcium, peroxidases are effectively inactive. Based on our results, lower concentrations of calcium led to an increase in enzyme activity in the first 1 hour. Moderate stability of the enzyme was observed at room temperature, at concentrations of 0.01 and 0.001 mg/mL for the first 24 hours, and at refrigerator temperatures, at a concentration of 0.001 between 24 and 72 hours.

In the present study, practical results were obtained regarding the thermal stability of the peroxidase enzyme in the presence of calcium. However, our study has limitations. The peroxidase enzyme was not purified, and a crude extract was used to study its activity. Further studies are needed to determine the exact effect of nanoparticles and calcium ions on the enzyme conformation and to study how they bind to the peroxidase enzyme.

## Conclusion

Considering the effect of calcium ions in increasing enzyme activity, it is expected that their use in combination with Calaminta officinalis Muench extracts can stabilize the enzyme and increase its activity. However, further research, such as purified enzyme studies, structural studies, and combination with other stabilizing agents, is needed to complete the results of this project.

## Abbreviation

ZnO NPs: Zinc oxide nanoparticles; POD: Peroxidase enzyme; COM: Calamintha officinalis Moench; SOD: Superoxide dismutase; CAT: Catalase; POD: Peroxidase

## Statements and Declarations

## Funding support

This study was not funded by any organization.

## Competing interests

The authors declare there is no Competing interests

## Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki.

## Consent to participate

Informed consent was obtained from all individual participants included in the study.

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