Evaluation of antiviral activity of ZnONPs using *Origanum vulgare* L. aqueous extract against Newcastle disease virus *in ovo*

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ABSTRACT

Introduction: Newcastle disease (ND), caused by the Newcastle disease virus (NDV), is a major viral threat to poultry, leading to high morbidity, mortality, and economic losses. Traditional prevention through biosecurity and vaccination is often insufficient, especially in areas with dense poultry populations. As a result, researchers are exploring alternative methods, such as natural antiviral compounds. The aim of present study was to assess the antiviral potential of zinc oxide nanoparticles (ZnONPs) synthesized using the aqueous extract of *Origanum vulgare* against NDV in embryonated chicken eggs. **Materials & Methods:** A total of ninety eggs were used and randomly divided into six experimental groups. Groups 1 to 4 received a combination of a velogenic strain of NDV and different concentrations (50, 100, 200, and 400 µg/ml) of the plant-based ZnONPs via the allantoic

route. Group 5 was inoculated with the virus alone as a positive control, while group 6 received only sterile phosphate-buffered saline as a negative control. Embryo viability was monitored daily, and the allantoic fluids were harvested for hemagglutination assays to detect the presence of NDV. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test to determine significant differences among treatment groups ($p < 0.05$). **Results:** The findings revealed that embryos treated with *Origanum vulgare* ZnONPs showed a markedly higher survival rate (20%, 60%, 80%, and 100% viability in 50, 100, 200, and 400 µg/ml treatments, respectively) compared with the positive control group. Additionally, virus titers in treated groups were significantly ($p < 0.05$) reduced (HA titer 10± 0.23 in the positive control group and HA titer 7 ± 0.20 in the 400 µg/ml group). **Conclusion:** The study strongly suggests that ZnONPs derived from *Origanum vulgare* extract possess promising antiviral activity against NDV in ovo, offering a potential complementary strategy for ND control.

Keywords: Hemagglutination (HA) titer, *In ovo*, Nanoparticles, Newcastle disease virus, *Origanum vulgare* L.

1. Introduction

Poultry production is the second largest agricultural industry in Iran and plays a vital role in supplying the protein requirements of the population. Similar to other countries, this industry faces numerous challenges from infectious (viral, bacterial, etc.) and non-infectious agents. Among these, Newcastle disease (ND) represents a major threat to poultry health, leading to substantial economic losses. The Newcastle disease virus (NDV) is classified by the International Committee on Taxonomy of Viruses as belonging to the species

Orthoavulavirus javaense, within the genus Orthoavulavirus, and the family Paramyxoviridae, and it induces gastrointestinal, respiratory, and neurological symptoms in affected birds [1]. Despite the routine vaccination of poultry flocks against NDV, vaccine failures occasionally occur due to factors such as breaches in biosecurity, viral mutations, or the presence of immunosuppressive agents [2]. Therefore, developing alternative or complementary strategies is essential to address these challenges and ensure the sustainability of the poultry industry.

In recent years, the use of natural products with antimicrobial and antiviral properties has gained increasing attention. Among these, *Origanum vulgare* L. has emerged as a versatile medicinal plant traditionally used for the treatment of colds, coughs, and digestive disorders. The *Origanum* genus is known to be rich in diverse bioactive compounds, including phenolics, lipids and fatty acids, flavonoids, and anthocyanins [3].

Nanotechnology is one of the most important fields of expanding research, especially about antiviral agents. Nanotechnology is the engineering and manufacturing of materials on an atomic and molecular scale and usually refers to structures whose size is up to several hundred nanometers, which are more mobile due to their small size and are suitable for nanotechnology [4]. The use of toxic chemicals as reducing agents in the synthesis of nanoparticles causes several side effects. During green synthesis, materials derived from biological sources, such as plant extracts, microorganisms, and biological waste, are applied. This method was developed because conventional synthesis involves hazardous chemicals, high energy use, and high costs, while biosynthesis offers an eco-friendly and affordable

alternative [5]. Studies have shown that zinc oxide nanoparticles can have antiviral properties as a biologically active compound [6].

In spite of having antioxidant, antibacterial, and antifungal activity, studies on antiviral activity of *Origanum vulgare* are still limited. To the best of our knowledge, this is the first study to investigate the green synthesis of zinc oxide nanoparticles (ZnONPs) using aqueous extract of *Origanum vulgare* L. and evaluate their antiviral activity against NDV.

2. Materials and Methods

2.1. Preparation of *Origanum vulgare* L. leaf extract

Origanum vulgare L. was obtained from the Behshahr (36° 41′, N, 53° 32′ E) in Mazandaran province, Iran. Dried leaves were powdered using a mixer. 8 grams of fine powder was dissolved in 150 mL of distilled water and blended for 50 min at 70 °C. The flask was incubated in the shaker at 25 °C for 72 hours then, centrifuged at 6000 rpm for 20 min. The final extract was collected using Whatman No.1 filter paper for filtration [7].

2.2. Synthesis of ZnO nanoparticles using *Origanum vulgare* L. leaf extract

The *O. vulgare* aqueous extract was successfully utilized to prepare ZnO nanoparticles, following the method described by Sharma et al. [8] with minor modifications. In this method, a 0.1 M Zinc acetate dehydrates solution was prepared in deionized water. 40 mL of the aqueous leaf extract was slowly added (dropwise) to a well-mixed solution of ZnO (160 mL). Then, it was placed on the stirrer at room temperature for 30 minutes, and then the solution pH was adjusted to 11 using sodium hydroxide (NaOH), then it was stirred at 80 °C

for 4 hours. In the following it was centrifuged for 20 minutes at 6000 rpm and washed three times with distilled water.

2.3. Characterization of ZnO nanoparticles

To corroborate the formation of nanoparticles, UV–Vis spectroscopy is commonly employed as one of the most essential and accessible techniques. The primary characterization of the ZnO nanoparticles was performed using UV–Vis spectroscopy. A double-beam UV–Vis spectrophotometer (Hanon i3, China), operating within the range of 300–600 nm, was utilized to confirm the production of ZnO nanoparticles synthesized using the leaf extract of *Origanum vulgare* L. X-ray diffraction (XRD) analysis (Bruker AXS D8 Advance, Germany) was carried out by the powder diffraction method over a 2θ range of 1°–80°. Surface morphology and particle distribution of the ZnO nanoparticles were examined using a field emission scanning electron microscope (FESEM) (TESCAN Vega3, Czech Republic).

2.4. Virus characteristics and Embryonated chicken eggs (ECEs)

A virulent field isolate of NDV (GenBank accession number: MK659694; ICPI: 1.72) was utilized as virus stock source [9]. In addition, all experiments were carried out using non-infected embryonated chicken eggs (ECEs) free of NDV-specific antibodies.

2.5. Extract Biosafety

The toxicity of the plant extract on egg embryos was investigated. For this purpose, dilutions of 10, 50, 100, 200, 400, and 800 µg/ml of the extract were prepared and, 100 µL of each concentration was injected into the allantoic cavity of 7-day-old ECEs. Then, the eggs were incubated at 37 °C for two weeks. The maximum dose of the extract that was not toxic to the eggs and hatched chicks were alive and healthy (400 µg/mL), was considered as the

maximum non-toxic concentration (MNTC), along with three lower concentrations (50, 100, and 200 µg/ml) used in the *in ovo* antiviral screening test. The Animal Ethics Committee of Amol University of Special Modern Technologies approved the test protocol (Ir. ausmt. rec. 1404.3).

2.6. Embryo Infectivity (EID_{50})

0.1 mL of NDV stock was propagated 72 hours in the allantoic cavity of 10-day-old chicken eggs at 37 °C. For accurate titer calculation, five eggs were used for each 10-fold dilution. The 50% egg infective dose (EID_{50}) titers were assessed by performing a serial dilution of viruses in eggs, and endpoints were calculated by Reed and Munch [10] formula. The EID50 titer of the virus strain was calculated to be 10^{-6} /0.1mL.

2.7. Inoculation of Eggs

The assay was carried out according to the method detailed by Bakari et al. [11] with some modifications. The study involved ninety 9-day-old embryonated chicken eggs (ECEs), which were randomly allocated into six equal groups (15 eggs per group) as follows: Groups 1–4 were inoculated with a mixture of virus (0.1 mL) and extract at concentrations of 50, 100, 200, and 400 µg/mL, respectively; Group 5 (positive group) was inoculated with 0.1 mL virus suspension, and Group 6 (negative group) was inoculated with 0.1 mL sterile phosphate-buffered saline. All injections were done via the allantoic route.

2.8. *In ovo* antiviral assay

Following the injection, the eggs were placed in the incubator at a temperature of 37 °C. Daily candling if the eggs was conducted to assess embryonic viability by observing embryo

movement and blood vessel development. Embryos that died within 24 hours were removed and replaced.

2.9. Hemagglutination test

After 72 hours, the eggs were transferred to the refrigerator and chilled at 4 °C. Subsequently, allantoic fluids were harvested from the eggs to perform the rapid hemagglutination test for NDV detection, in accordance with the protocol outlined by Murakawa et al. [12].

2.10. Statistical analysis

Embryo viability among experimental groups was evaluated using SPSS software version 23. The differences were analyzed by analysis of variance (ANOVA) and the standard deviation ± mean (Mean ± SD) was used to compare the means through post hoc testing with Duncan's multiple range test. Statistical significance was established at $P < 0.05$.

3. RESULTS

3.1. Characterization of the biosynthesized ZnO nanoparticles

There are several techniques to confirm the synthesis of nanoparticles. As depicted in Figure 1, the existence of biologically synthesized ZnONPs was confirmed in the range of 200-700 nm by observing the maximum absorption peak at 360 nm. Our findings are consistent with previous studies that show a characteristic absorption peak range of 330-460 nm [13]. The XRD pattern of bio-synthesized ZnO nanoparticles from the leaf extract of *Origanum vulgare* L. is shown in Figure 2. The sample was analyzed at a range of angles from 0° to 100°. The prominent x-ray diffraction peaks exhibited by ZnO nanoparticles are at $2\theta = 31.84°$, 34.56°, 36.38°, 47.72°, 56.73°, 62.96°, 66.73°, 68.05°, 69.27°, 72.64°, and 77.07°, and could be indexed as (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202),

158 respectively. The XRD pattern revealed the orientation and crystalline nature of ZnO-NPs.
159 Sharp and narrow diffraction peaks imply the pure crystalline nature of the nanoparticles.
160 The results of Faisal et al. [14] showed the same diffraction peaks while examining the XRD
161 patterns of green-synthesized ZnO nanoparticles with the aqueous extract of *Myristica*
162 *fragrans*, which confirmed our results. ZnO nanostructures have been observed in various
163 forms such as nanospheres, nanorods, and others. FESEM is used to imagine and analyze
164 every small topographic detail and is accordingly used to assign the particle dimensions and
165 morphology. The results of FESEM micrograph observations in Figure 3 at a resolution of
166 200 nm showed that the morphology of biosynthesized zinc oxide nanoparticles is spherical
167 [15].
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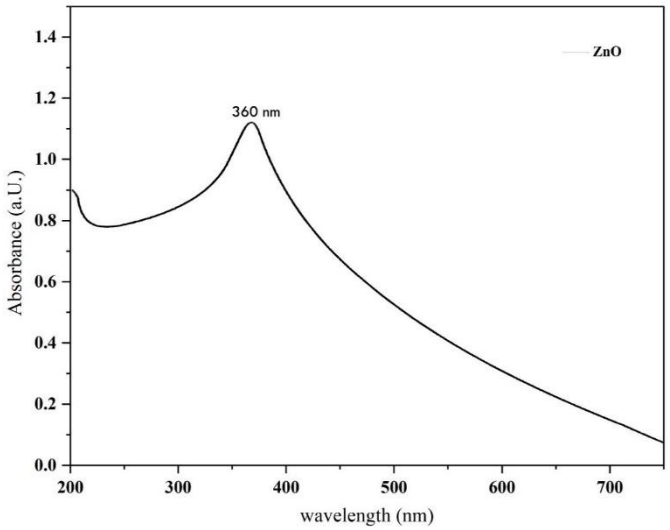

Figure 1. Visible ultraviolet spectroscopy of synthesized zinc oxide nanoparticles using *Origanum vulgare* aqueous extract

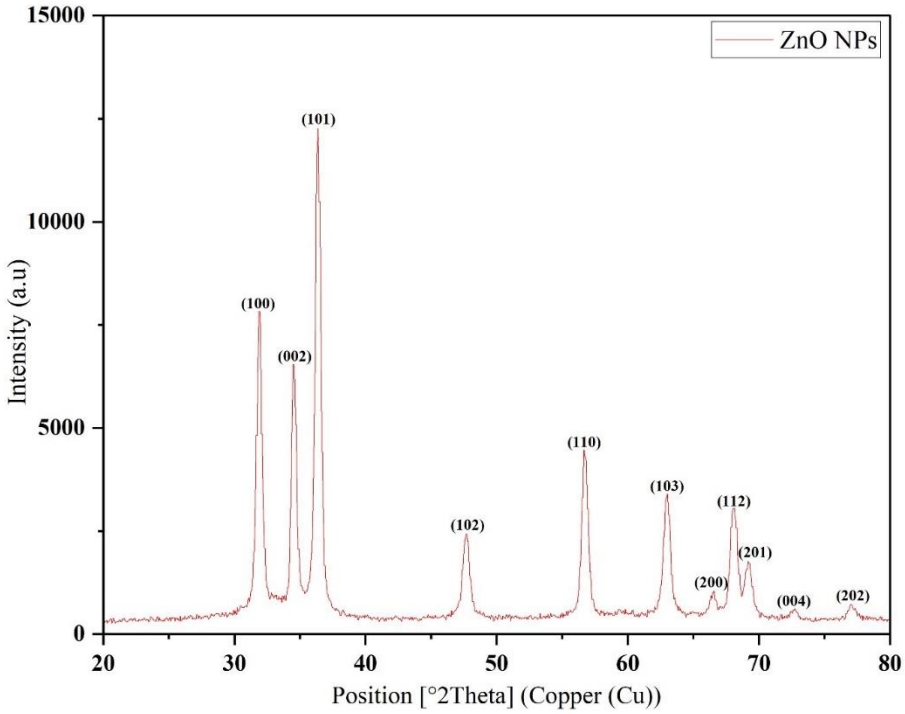

Figure 2. X-ray diffraction (XRD) pattern of the zinc oxide nanoparticles

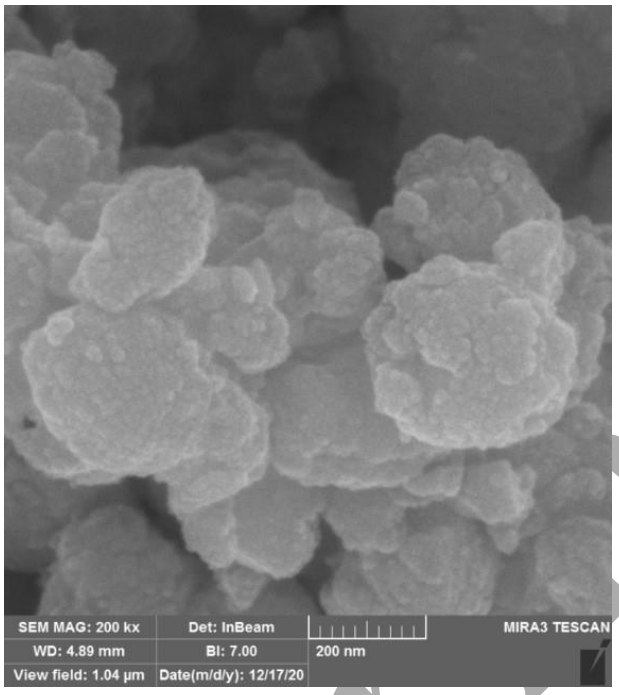

Figure 3. FESEM images of zinc oxide nanoparticles

3.2. Effect of treatments on embryo viability

Results related to embryo death times are shown in Table 1.

Table 1. Antiviral activity of ZnO-NPs of *Origanum vulgare* extract on Newcastle disease

Treatment	Concentration µg/mL	Number of Eggs	Mortality with different time intervals			% Mortality
			24 hours	48 hours	72 hours	
ZnO-NPs of *Origanum vulgare*	50	15	0 ± 0.0	10 ± 0.34	2 ± 0.08	80
	100	15	0 ± 0.0	9 ± 0.29	0 ± 0.0	60
	200	15	0 ± 0.0	3 ± 0.15	0 ± 0.0	20
	400	15	0 ± 0.0	0 ± 0.0	0 ± 0.0	0
PC	-	15	0 ± 0.0	15 ± 0.42	0 ± 0.0	100
NC	-	15	0 ± 0.0	0 ± 0.0	0 ± 0.0	0

virus

PC: Positive control, NC: Negative control

No mortality was observed in experimental groups 24 hours post-incubation. The positive control group experienced complete mortality of all embryos after 48 hours of incubation, with allantoic fluid samples yielding positive results in the rapid hemagglutination test. In contrast, the negative control group showed no instances of mortality, and all allantoic fluid samples tested negative for hemagglutination. The addition of nanoparticles of *Origanum vulgare* extract extended ($P < 0.05$) the embryo survival time in a dose-dependent manner. The application of 400 µg/mL led to a complete inhibition of NDV replication, with no recorded mortality. Also, a concentration of 200 µg/mL resulted in a 20% mortality rate, suggesting that while this dosage can control the virus, it is less effective than the higher concentration ($P < 0.05$ as compared to the positive group). High mortality rates (80% and 60%) were observed in 50 and 100 µg/mL groups, respectively. Figure 4 shows gross pathology in the treatment groups. Embryos in the positive control group were dwarfed and congested, with sub-cutaneous hemorrhages in the head and body, whereas no lesions were seen in the negative control or ZnO-NPs (400 µg/mL) groups.

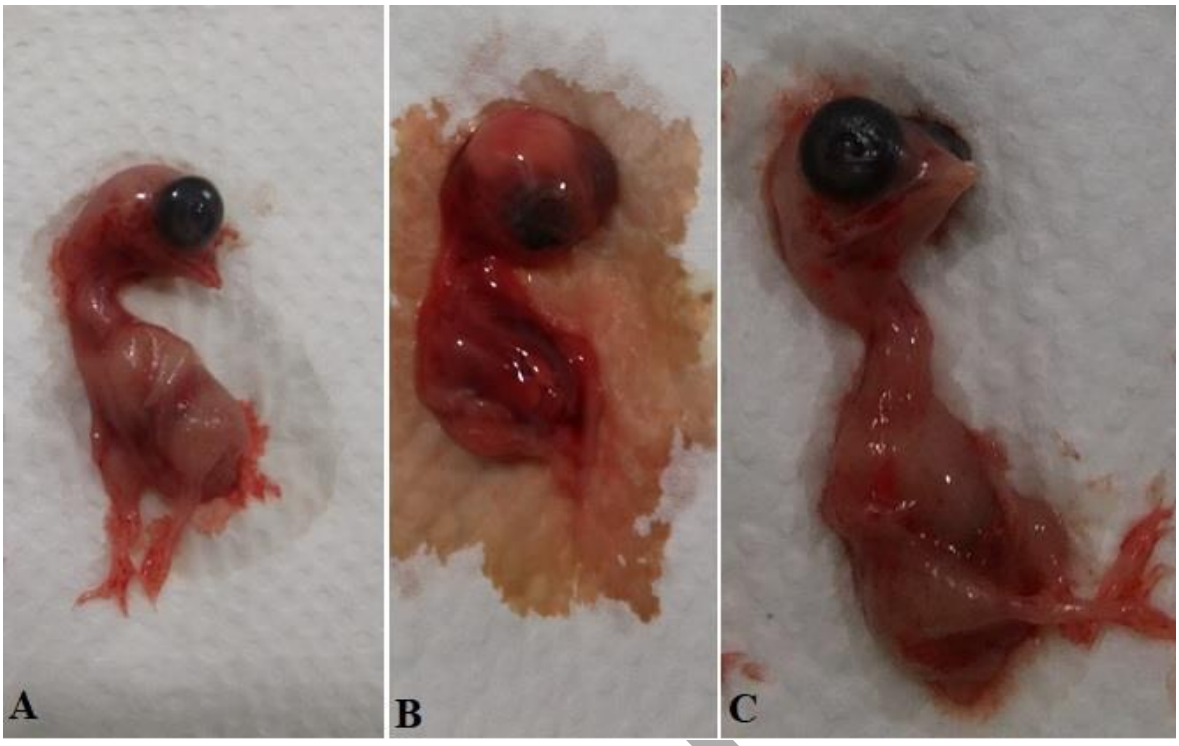

Figure 4. Lesions on chicken embryos in treatment groups. A) Negative control B) positive control with dwarfism, congestion, and sub-cutaneous hemorrhages C) ZnO-NPs using *Origanum vulgare* aqueous extract (400 µg/mL) group

3.3. Effect of treatments on HA titer

Table 2 shows the HA titers of chicken embryos infected with NDV following treatment with different concentrations of *Origanum vulgare* L. extracts nanoparticles. The results indicated that all extracts effectively reduced HA titers depending on their doses. NDV was not detected in the negative control (HA: 0 ± 0.00), while the highest HA titer value (10 ± 0.23) was observed in the positive control. In experimental extract groups, the lowest HA titers (7 ± 0.19 and 7 ± 0.20) were recorded in eggs inoculated with NDV and extracts at doses of 200 µg/mL and 400 µg/mL.

Table 2. Mean hemagglutination (HA) titers in embryonated chicken eggs inoculation with NDV and different concentrations of ZnONPs of *Origanum vulgare* extract

Treatment	Concentration µg/mL	Average HA (Log_2) titer
ZnO-NPs of *Origanum vulgare*	50	9 ± 0.18
	100	8 ± 0.15
	200	7 ± 0.19
	400	7 ± 0.20
PC	-	10 ± 0.23
NC	-	0 ± 0.00

PC: Positive control, NC: Negative control

4. DISCUSSION

The present study has demonstrated the antiviral properties of ZnO-NPs of *Origanum vulgare* L. extract against the Newcastle disease virus. We observed that all chicken embryos inoculated with the NDV died within two days, indicating the highly virulent characteristics of the virus strain. Nevertheless, the survival duration of the chicken embryos was significantly extended in the groups treated with the extract, in a dose-dependent manner. These results suggest that ZnO-NPs of *Origanum vulgare* may offer a viable complementary

strategy for managing NDV in poultry, particularly in regions where conventional biosecurity and vaccination measures are insufficient.

The antiviral potential of plant-derived extracts against Newcastle disease virus (NDV) has been widely documented. Andleeb et al. [16] demonstrated the inhibitory activity of *Iresine herbstii* extract against NDV. Similarly, *Illicium verum* extracts have been reported to suppress the replication of several avian viruses, including avian reovirus, infectious bursal disease virus (IBDV), NDV, and infectious laryngotracheitis virus (ILTV) [17]. Bakari et al. [11] also reported that *Commiphora swynnertonii* extracts exhibited significant antiviral activity against NDV in ovo. Furthermore, the inhibitory effects of aqueous garlic extract on a highly pathogenic field isolate of NDV have been evaluated, confirming its potential as a natural antiviral agent [18].

The concentration and composition of bioactive constituents in medicinal plants are influenced by multiple environmental factors, including geographical origin, seasonal variation, and the climatic and ecological characteristics of the collection site. Previous studies have reported that numerous plant species traditionally employed in the treatment of viral diseases contain substantial amounts of secondary metabolites such as alkaloids, terpenes, flavonoids, naphthoquinones, coumarins, and anthraquinones. These compounds exhibit antiviral properties primarily through direct virucidal effects or by interfering with various stages of viral replication. In the case of Newcastle disease virus (NDV), the key surface glycoproteins—hemagglutinin-neuraminidase (HN) and fusion (F) proteins—are essential for viral attachment to host cells and subsequent replication. Certain phytochemical constituents have demonstrated protease inhibitory activity, which may impede the cleavage of these glycoproteins, thereby blocking viral entry and reducing replication efficiency [19].

According to the results of our study, virus HA titers was decreased by *Origanum vulgare* extract. Therefore, *Origanum vulgare* extract may have affected the reproduction of the virus, resulting in a decrease in the number of viral particles. Research has indicated that the presence of protease-inhibitory activity can be found in various plant species. Protein kinase C (PKC) from rat brain was inhibited by plant flavonoids, and some flavonoids, such as fisetin, quercetin, and luteolin, had more inhibitory effects [20]. These compounds could interfere with the cleavage of glycoproteins and inhibit virus adhesion [11]. Quercetin, one of the flavonoids present in the extract of *Origanum vulgare*, has been reported to exhibit antiviral activity. It has been proposed that the antiviral activity of quercetin has been attributed to its capacity to bind with viral envelope glycoproteins and thus interfere with virus adsorption and cell entry and also interfere with DNA synthesis [21].

The observed decreases in virus populations within extract-treated embryos, which occurred in a dose-dependent manner, indicated a significant viricidal effect. This was revealed by the complete inhibition of virus growth *in ovo* at 400 mg/ml. At this concentration, all the inoculated eggs had live embryos. These findings align with the outcomes of previous studies investigating the efficacy of medicinal plant extracts in combating Newcastle disease virus. For instance, Bhuvaneswar et al. [22] evaluated the antiviral activity of the methanolic root extract of *Sophora interrupta* Bedd. against the Newcastle disease virus. Antiviral activity of *Achillea millefolium* and *Thymus vulgaris* extracts against Newcastle disease virus in ovo was reported [23]. Additionally, a recent study by Credo et al. [24] stated the anti-Newcastle disease virus activity of *Synadenium glaucescens* Pax leaves.

One critical aspect of using medicinal plant extracts is their typically low stability, which can limit their effectiveness and shelf-life. The medicinal properties of plants stem from secondary metabolites, including phenolic compounds, terpenoids, and nitrogen-containing cyclic compounds, which are generally polar and soluble in nature. These compounds often face challenges in passing through cell membranes passively due to their size or limited solubility in fats, resulting in poor bioavailability and vulnerability to degradation under unfavorable conditions (e.g., oxygen, temperature, pH). To improve the absorption and bioavailability of these active plant constituents, various delivery systems, especially nanocarriers, are utilized. Nanoparticle coating help these compounds traverse biological membranes and blood barriers more efficiently. Moreover, their nanoscale size minimizes detection and elimination by the body's defense mechanisms, like the kidneys and liver, thus extending the duration of the compound's effects and lowering the necessary dosage [25]. For this reason, in the present study, the green synthesis of zinc oxide nanoparticles (ZnONPs) using the aqueous extract of *Origanum vulgare* L. and their evaluation against NDV was investigated.

5. CONCLUSION

The results of the antiviral assay for ZnO-NPs of *Origanum vulgare* highlight its potential as an effective agent against the Newcastle disease virus. Further investigation is advised to discover the beneficial compounds found in this plant, to utilize them for therapeutic applications and preventive strategies in the future.

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Authors' Contribution

Study concept and design: S. S., M. KH.

Analysis and interpretation of data: M. G., S. M.

Drafting of the manuscript: S. S., M. G.

Acquisition of Data: M. KH., S. M.

Critical revision of the manuscript for important intellectual content: S. S., M. G.

Study Supervision: S. S., M. KH.

Ethics

We declare that all ethical standards related to animal health and welfare were respected in the present study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Data Availability

The data supporting the findings of this study are available upon request from the corresponding author.

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