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Received: 18 September 2025

Accepted: 8 January 2026

Published online: 28 January 2026

Cite this article as: Durna Ö., Ulufer Bulut S., Boy S. *et al.* In vitro characterization and in ovo embryotoxicity assessment of a triazol-5-one derivative in broiler embryos. *Sci Rep* (2026). <https://doi.org/10.1038/s41598-026-35880-0>

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In vitro Characterization and In ovo Embryotoxicity Assessment of a Triazol-5-one Derivative in Broiler Embryos

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Abstract

This study is among the pioneering investigations evaluating the embryonic effects of triazol-5-one derivatives administered in ovo to broiler embryos. The synthesized compound, 1-(morpholin-4-yl-methyl)-3-(*p*-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one, was examined for its in vitro biological properties and in ovo embryonic safety. The compound is hypothesized to influence embryonic viability in a manner consistent with its structural and biological characteristics. Structural confirmation was achieved through FT-IR, ¹H-NMR, and ¹³C-NMR analyses. In vitro assays showed weak reducing and radical-scavenging capacity but high, stable metal-chelating activity, alongside strong antimicrobial effects. For the in ovo trial, 120 fertilized Ross-308 eggs were assigned to three groups (Control, Dimethyl sulfoxide (DMSO), Experimental; n=40). On day 17, 0.5 mL of solution was injected into the amniotic cavity, and embryos were monitored until 510 hours. Hatchability was 87.5% in the Control group, 80% in the DMSO group, and 0% in the Experimental group. No live chicks hatched in the experimental group, indicating complete embryonic lethality. All embryo losses occurred after injection during incubation, and Levene's test followed by one-way ANOVA (p=0.385) confirmed no initial weight differences, indicating a compound-specific effect. The compound's embryotoxicity may result from strong metal chelation, low antioxidant capacity, oxidative stress, mitochondrial dysfunction, and membrane disruption. These findings provide a basis for defining

safe dose ranges, optimizing injection parameters, and assessing the in ovo applicability of related triazole compounds.

Keywords: Broiler chicken; in ovo injection; embryotoxicity; hatchability; triazole compound

Introduction

Advances in pharmaceutical research have increased interest in chemically versatile heterocyclic systems, bringing 1,2,4-triazole derivatives into prominence (Abdelli et al., 2021; Korol et al., 2023). The antioxidant (Pachuta-Stec, 2022), antimicrobial (Tian et al., 2023), and anti-inflammatory (Szczukowski et al., 2021) potential of triazole derivatives largely stems from their enzyme-inhibitory properties and free-radical scavenging capacity. Several studies have demonstrated that methoxy- and hydroxyl-substituted derivatives effectively modulate oxidative stress and inflammatory pathways (Aggarwal et al., 2022; Kumari et al., 2021; Majage et al., 2025). In addition to these mechanisms, triazole-based compounds are known to exhibit strong antifungal (Sadeghian et al., 2023) and antiparasitic (Rezali et al., 2025) effects. These biological properties highlight the relevance of triazole derivatives for animal health, including potential applications in poultry production such as pathogen control and reduction of microbial contamination in poultry environments. However, despite their potential applicability, no data exist on how triazole derivatives interact with avian embryos, making it essential to evaluate their embryonic safety before any practical use can be considered.

In avian species, embryonic development is a tightly regulated process characterized by rapid metabolic transitions, increasing oxygen demand, and progressive differentiation of vital organs. The late incubation period, particularly days 15–19 in broilers, represents a physiologically sensitive window in which even minor metabolic disturbances may impair viability (Ghanaatparast-Rashti et al., 2018; Kpodo and Proszkowiec-Weglarz, 2023). The in ovo technique provides direct access to the embryonic environment, enabling controlled delivery of bioactive substances without inducing post-hatch handling stress (Gupta et al., 2022.) Therefore, understanding how pharmacological or antioxidant-active compounds interact with avian embryos is essential for evaluating both safety and potential biological utility.

Despite their widespread use as antioxidant, antimicrobial, anti-inflammatory, antifungal, and antiparasitic agents, the embryonic safety of triazole derivatives in avian species has never been evaluated, creating a critical knowledge gap. Furthermore, studies evaluating 1,2,4-triazole derivatives in vivo using experimental animal models have shown that metabolic responses to these compounds can vary markedly among species, and that the clinical relevance of such findings remains limited unless these interspecies differences are clarified. In this context, rodent studies conducted by Al-Mansury et al. (2022), Azim et al. (2021), Oruç et al. (2003), and Timur et al. (2019) reported that 1,2,4-triazole derivatives have been reported to influence antioxidant enzyme activities, anti-inflammatory and antimicrobial responses, as well as broader systemic physiological reactions. Conversely, Gupta et al.

(2022) demonstrated that in ovo administration of α -ketoglutaric acid is safe for broiler embryos and improves antioxidant status and metabolic parameters. However, the absence of any studies evaluating 1,2,4-triazole derivatives in poultry species such as broiler chicks reveals a notable gap in the existing literature and emphasizes the necessity for research in this field. These findings support the premise that the in ovo technique represents a biologically valid model for evaluating antioxidant-active or pharmacologically potent compounds during avian embryogenesis. Taken together, the current body of evidence suggests that 1,2,4-triazole derivatives hold significant potential for investigation in broiler embryos and may provide valuable alternatives for poultry production systems.

The biological properties of triazole derivatives suggest that they may hold potential relevance for poultry production systems, particularly in pathogen control and feed preservation. Considering the practical limitations and stress-inducing effects of injection-based systemic applications in poultry, the in ovo feeding approach has become a preferred alternative due to its ability to deliver substances directly into the embryonic environment and its suitability for integration with field applications (Tako et al., 2004; Peebles, 2018). It has been reported that vitamin E, zinc, probiotics, and amino acids can be successfully transferred via in ovo applications, thereby improving growth performance, intestinal development, and immune activation (Ohta et al., 1999; Araújo, 2019; Ahmed et al., 2023). It is also considered that this method may enable the evaluation of bioavailability and safe dose profiles for pharmacological agents such as triazole derivatives, providing a critical starting point for establishing their embryonic safety profiles.

Current literature indicates that various bioactive compounds administered in ovo, such as prebiotics, organic acids, and certain minerals, can exert notable effects on growth performance and immune responses. For instance, Bednarczyk et al. (2016) reported that prebiotics including β -glucan and galactooligosaccharides increased body weight during the first 21 days post-hatch. Similarly, Gupta et al. (2022) demonstrated that in ovo administration of 1% α -ketoglutaric acid enhanced daily weight gain and feed intake in broilers. Mineral-based interventions have also been explored, with iron supplementation increasing body weight by day 42, whereas copper did not produce a comparable effect. Nevertheless, literature analyses reveal that studies on in ovo feeding have predominantly focused on nutritional or immunological components (Hou & Tako, 2018; Das et al., 2021), while largely overlooking pharmacological heterocyclic derivatives. Consequently, no systematic evidence exists regarding the in ovo use of 1,2,4-triazole derivatives during embryonic development, despite their strong antioxidant and enzyme-inhibitory properties demonstrated in rodent models.

To address this gap, the present study aims to evaluate the in ovo applicability of a 1,2,4-triazole derivative in broiler embryos and, in the event that hatching occurs, to assess the compound-associated embryonic and post-hatch physiological effects in detail. In line with this objective, the structural features of the synthesized 1,2,4-triazole derivative will be confirmed, followed by in vitro assessment of its antioxidant and antimicrobial properties prior to its in ovo administration. Previous findings

indicate that the success of in ovo strategies is critically dependent not only on the type, dose, and timing of the administered compound but also on factors such as injection volume and site, solvent toxicity profile, embryonic developmental stage, and procedural sterility (Bednarczyk et al., 2016; Das et al., 2021; Gupta et al., 2022; Hou & Tako, 2018). The full compliance of the in ovo protocol used in this study with the standard procedures described in the literature (Kpodo and Proszkowiec-Weglarz, 2023; Peebles, 2018; Zhai et al., 2011) allows the biological characterization of the compound to more reliably determine whether the observed reduction in hatching success, or the complete absence of hatching, originates from the compound itself rather than from methodological factors.

The primary objective of this study was to evaluate the in ovo applicability and embryonic safety of a *p*-chlorobenzyl-substituted triazol-5-one derivative, supported by structural confirmation and in vitro biological characterization. Within the scope of the study, the triazol-5-one derivative was anticipated to exert measurable effects on embryonic development, hatchability, and early post-hatch physiological responses. Accordingly, the synthesized compound 1-(morpholin-4-yl-methyl)-3-(*p*-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one was evaluated using both in vitro and in ovo approaches. Considering the literature reporting that triazole derivatives may exhibit a high affinity for metal ions, interact with antioxidant defense systems, and possess the potential to disrupt certain cellular structures, it is plausible that embryo viability may be adversely affected following in ovo administration. On the other hand, several studies have emphasized that hatchability is highly sensitive to factors such as the concentration of the administered compound, injection volume, solvent toxicity, timing of application, and the developmental stage of the embryo. These reports highlight that reductions in hatchability during in ovo procedures may arise not only from the bioactive compound itself but also from various procedural or chemical factors (Oliveira et al., 2023; Araújo et al., 2019). Therefore, in the present study, all potential variables were carefully considered, and the in ovo protocol was fully aligned with the standard methods described in the literature. The synthesized *p*-chlorobenzyl-substituted triazol-5-one derivative was subjected to both structural confirmation analyses and comprehensive biological characterization. Furthermore, should hatching occur, detailed evaluations will be carried out to assess serum biochemical responses associated with fasting and transfer stress inherent to the in ovo procedure, as well as potential alterations in liver function indicators and biological changes in pectoral muscle tissue.

It was hypothesized that the synthesized triazol-5-one derivative may alter embryonic viability when administered in ovo due to its structural and biological characteristics. From a broader perspective, examining the *p*-chlorobenzyl-substituted triazole compound through an in ovo approach has the potential to contribute not only to fundamental scientific knowledge but also to sectoral efforts aimed at developing sustainable and antibiotic-free strategies in poultry production. Even in the absence of successful hatching, the experimental data generated in this study may guide the determination of more appropriate dose ranges, the optimization of injection parameters, and the refinement of formulation

strategies in future research, and may further pave the way for in ovo investigations involving triazole compounds that are structurally similar or chemically distinct. Conversely, should hatching occur, the physiological responses elicited by the compound may provide an alternative scientific basis for developing pharmacological intervention strategies in broilers subjected to stress conditions.

Materials and Methods

Chemical Overview of the Synthesized Compound

The target *p*-chlorobenzyl-substituted triazol-5-one derivative was obtained through a conventional two-step synthetic sequence involving initial Schiff base formation followed by a Mannich condensation. The structural identity and purity of the compound were confirmed by FT-IR, ¹H-NMR, and ¹³C-NMR spectroscopic analyses. Detailed reaction conditions, purification procedures, the reaction scheme (Supplementary Material, Scheme S1), and complete spectroscopic data (Supplementary Material, Table S2) are provided in the Supplementary Material.

Bioefficacy Screening of Triazole Derivatives

Prior to inclusion in the in ovo injection protocol, the biological properties of the triazol-5-one derivative used in the study were subjected to a detailed in vitro evaluation. Three different methodological approaches were preferred in determining the antioxidant profile: electron transfer capacity (ETC), free radical neutralization power (FRN), and metal ion chelation capacity (MIC).

The ETC analysis was performed according to the Oyaizu (1986) method; in this method, the reducing potential of the compound was measured at 700 nm by relating it to the absorbance value of the Prussian blue complex. In the FRN test conducted using the DPPH radical, the radical scavenging effect of the compound was evaluated by the decrease in absorbance at 517 nm (Blois, 1958). Finally, the Dinis et al. (1994) method was used in the MCA analysis, and the chelation capacity was determined by absorbance measurements at 562 nm. α -Tocopherol, BHA, BHT, and EDTA were included as references for standard comparisons.

Additionally, the functional evaluation of the synthesized compound included an antimicrobial activity assessment using the Agar Well Diffusion Method. For this purpose, the compound was tested for its inhibitory potential against a panel of six bacterial strains selected due to their clinical and environmental relevance. These strains comprised three Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*) and three Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*) (Perez et al., 1990). Bacterial suspensions were standardized to a density of 10⁶ cfu/mL and inoculated onto Mueller–Hinton agar plates, after which 50 μ L of the test compound was dispensed into the wells. Ampicillin, Neomycin, and Streptomycin served as reference antibiotics, while DMSO was used as the solvent control. These biological assays ensured that the compound was

functionally assessed following structural verification and provided preliminary insights into its potential applicability in the in ovo system.

Laboratory Procedure and Implementation

In ovo Feeding Procedure

In ovo feeding studies, the combination of compounds yields positive results only under specific technical conditions. The chemical structure and bioactive potential of the compound used are among the key factors determining the physiological outcomes of in ovo feeding. Technical parameters are critical elements that directly shape both biological effects and safety.

Ghanaatparast-Rashti et al. (2018) reported that manual application of a combination of dextrin (20-40%) and β -hydroxy- β -methylbutyrate (HMB, 0.5-1%) to the amniotic fluid on the 18th day of incubation improved muscle glycogen levels and intestinal structure; but reported that high dextrin concentrations reduced hatchability. Gao et al. (2017) showed that in ovo L-arginine application at different doses had significant effects on digestive system development and growth performance, but hatchability decreased at high doses. On the other hand, Uni et al. (2005) stated that a 1.2 mL sugar solution injection increased liver glycogen, but this volume created a metabolic load in the embryo and negatively affected the hatch rate when the dextrin concentration exceeded 20%. Similarly, Zhai et al. (2011) reported that high application volumes of 1.2 mL reduced hatching, while Peebles (2018) emphasized that low volumes of 0.1-0.2 mL are safe in terms of embryo viability. When these data are evaluated together, it is understood that keeping the injection volume within the range of 0.1-0.6 mL is necessary to remain within embryonic tolerance limits and prevent metabolic overload.

Araújo et al. (2019) reported that in ovo administration of vitamin E at a level of 60.4 international units reduced oxidative stress and improved chick quality, but that the manual injection method has limited applicability on an industrial scale. Therefore, precise measurement and control of the dose, manual application, the use of high-purity compounds, and late embryonic timing (17-18 days) should be preferred (Kpodo and Proszkowiec-Weglarz, 2023). These studies demonstrate that not only the type of compounds but also the method and conditions of application directly affect the success of in ovo strategies.

Institutional Ethics Approval and Regulatory Compliance

Today, in ovo techniques aim to provide chicks with a metabolic advantage in their postnatal life by enabling targeted biological interventions during embryonic development. In commercial lines such as Ross 308, injections performed between the 17th and 19th days of incubation are considered the most appropriate timing because active absorption by the embryo occurs during this period. In this study, the amniotic cavity was selected as the injection site, and procedures were performed under aseptic

conditions. Micro-punctures made in the egg were closed using special techniques after injection, thereby minimizing the embryo's exposure to external factors.

While classical in ovo applications have focused on vitamin, mineral, and prebiotic formulations; this study presents a rare example in the literature of an application evaluating a synthetic compound such as 1-(morpholin-4-yl-methyl)-3-(*p*-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one using the in ovo method.

All experimental stages of the study were conducted with the approval of the Kafkas University Local Animal Experiment Ethics Committee (KAU-HADYEK/ 2019-086) and in accordance with the relevant legal regulations. Full compliance was ensured with the relevant articles (5/1a, 5/1b-3, 5/1c) specified in the regulation published in the Official Gazette dated February 15, 2014, and numbered 28914, in line with ethical practices in Turkey, and with the European Union's Directive 2010/63/EU. Furthermore, the animal welfare principles of the Ministry of Agriculture and Forestry were observed. The experimental design was organized in light of the 3Rs (Replacement, Reduction, Refinement) principles. Initially, 250 fertilized eggs were allocated to the study. From these, 120 eggs were assigned to the in ovo experimental groups (Control, DMSO, Experimental).

Egg Development and In Ovo Procedure

Fertilized eggs from the Ross 308 breed were supplied by Banvit Co. and used in the research conducted at the Kafkas University Prof. Dr. Ali Rıza Aksoy Education-Research and Application Farm. Eggs obtained from 35-42 week-old breeding animals were subjected to physical control; eggs with deformities, cracks, or contamination were eliminated. The remaining eggs were classified according to their weight and incubated. To prevent contamination, each egg was subjected to formalin fumigation in a 1 m³ area with 40 mL of 37% formaldehyde and 20 g of potassium permanganate (Hayretdağ and Kolankaya, 2008; Pagala and Has, 2024; Santos et al., 2025).

The incubation process was carried out according to classic standards: temperature was maintained at 37.7°C and humidity at 60% between days 0 and 18; from day 18 onwards, temperature was maintained at 37°C and humidity at 70%. On day 17, the eggs were examined under a lamp, eggs that had not completed embryonic development were separated, and the injection site was marked on the shell.

The study aimed to evaluate the in ovo applicability and embryonic safety of the compound 1-(morpholin-4-yl-methyl)-3-(*p*-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one. Three groups were formed: a non-injected control group, DMSO group treated only with the PBS–DMSO solution, and an experimental group treated with 15 mg of the active compound. Each group was designed to contain 40 eggs (total n = 120). To compensate for potential embryonic or handling losses, 250 eggs were initially allocated.

Due to the limited aqueous solubility of Compound **3**, a suspension rather than a solution was prepared for in ovo administration. For each egg, 15 mg of the compound was transferred into a sterile tube and pre-wetted with 3 μ L of DMSO, corresponding to 0.6%, to facilitate particle dispersion. The final volume was then adjusted to 0.5 mL with sterile Phosphate Buffered Saline (PBS, pH 7.3), which provides a physiologically compatible pH and ionic environment for embryonic tissues (Chen et al., 2021; Gopi et al., 2020). The buffering capacity of PBS maintained pH stability but did not fully dissolve the compound. Prior to injection, the mixture was vortexed for 30–60 seconds to minimize sedimentation and ensure a homogeneous suspension.

Injections were performed on day 17 using 0.5 mL solutions and 21G sterile needles. Amniotic fluid was selected as the target area. The area was sterilized with ethanol before application, and the puncture sites were covered with sterile tape after the procedure. Broiler chicken embryos have a standard 21-day incubation period, which corresponds to approximately 504–510 hours (Archer and Mench, 2014; Tainika and Bayraktar, 2022); accordingly, hatching was monitored at 510 hours, representing the full incubation duration from the beginning of incubation.

Previous research has demonstrated that injection volume is a critical factor influencing embryo viability in in ovo applications. Peebles (2018) reported that volumes in the range of 0.1–0.2 mL are considered safe for late-term embryonic procedures. In contrast, Uni et al. (2005) and Zhai et al. (2011) showed that high injection volumes such as 1.2 mL create a metabolic burden on the embryo and markedly reduce hatchability. When these findings are evaluated collectively, the 0.5 mL injection volume used in the present study remains well below the high-risk threshold identified in the literature and is therefore regarded as tolerable for late embryonic injection stages (E17–E19). Since broiler embryos were used in this study, the selected volume falls within the acceptable limits for this species and developmental period.

Statistical Analysis

Before incubation, fertilized eggs were randomly divided into three groups (Control, DMSO, Experimental) and egg weights for each group were weighed before incubation. Without performing significance tests, the homogeneity of variances was examined using the Levene test. Statistical analysis of recorded egg weights and hatchability was performed using one-way analysis of variance (ANOVA), and an appropriate post-hoc test (Tukey's test) was used to determine the significance of differences between groups. Statistical analysis was performed using the SPSS software package (IBM Inc, Chicago, USA, 2011).

Results

The successful synthesis of the compound was confirmed by FT-IR, ^1H -NMR, and ^{13}C -NMR analyses. The FT-IR spectrum displayed the characteristic O–H stretching at 3302 cm^{-1} , the triazole-associated

C=O band at 1703 cm^{-1} , and the C=N stretching of the Schiff base at 1597 cm^{-1} . In the ^1H -NMR spectrum, the benzylic CH_2 proton appeared at 4.09 ppm, the NCH_2N signal at 4.57 ppm, and the imine proton ($\text{N}=\text{CH}$) at 9.50 ppm. The ^{13}C -NMR spectrum exhibited characteristic signals at 144.54 and 150.33 ppm, corresponding to the triazole C3 and C5 carbons, along with the benzylic carbon at 49.99 ppm. Complete spectral data are provided in the Supplementary Material (Table S2).

Following structural confirmation, antimicrobial and antioxidant assays were performed to assess the biological activity of the compound. The reducing capacity measured at 700 nm at three concentrations is shown in Figure 1.

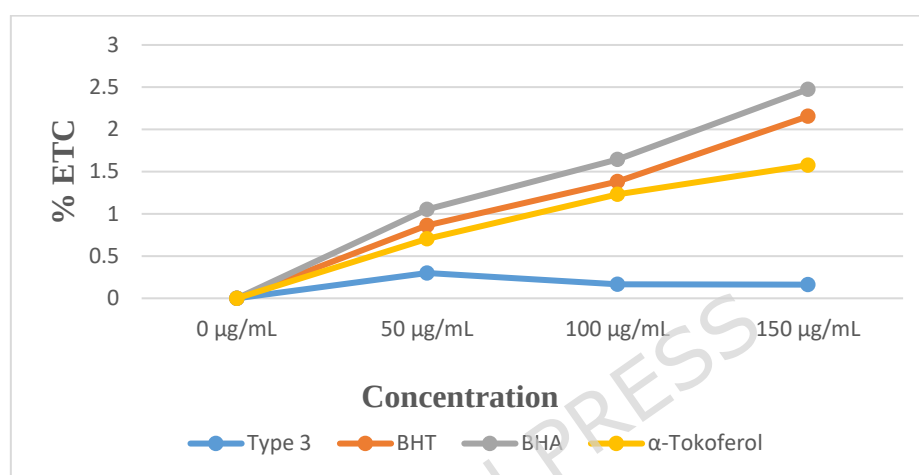


Figure 1. % ETC of Compound 3

The data presented in Figure 1 show that the reducing power of Compound 3 remained at low levels across all concentrations. Even at increasing concentrations from 50 $\mu\text{g/mL}$ to 150 $\mu\text{g/mL}$, the compound did not show a significant increase, and the absorbance values remained nearly constant. In contrast, the standard antioxidants BHA, BHT, and α -tocopherol showed a gradual increase depending on the concentration; BHA, in particular, exhibited the highest reduction activity. These findings indicate that the Compound 3 cannot exert a strong antioxidant effect through the reduction mechanism, but raise the possibility that it may exhibit different biological activities through other mechanisms.

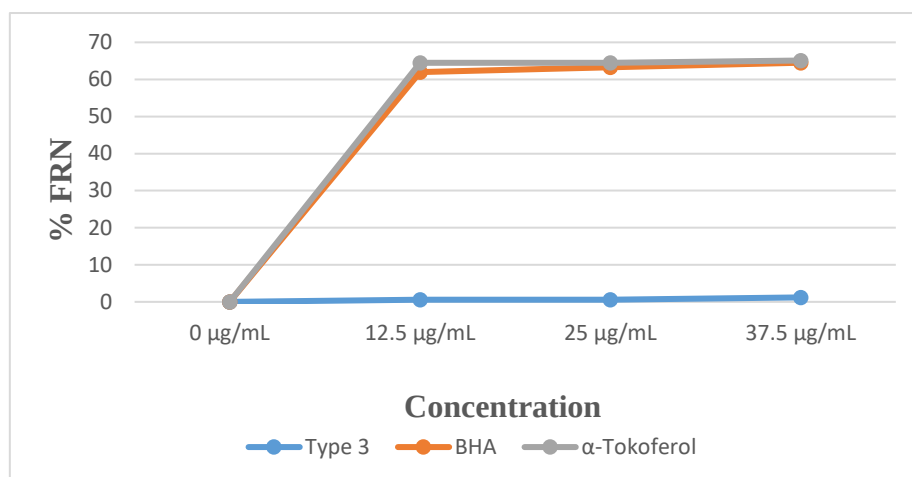


Figure 2. % FRN Activites of Compound **3**

Figure 2 shows that the free radical neutralization capacity of Compound **3** is negligible. At increasing concentrations, BHA and α -tocopherol exhibit a strong radical scavenging effect at levels of 60–65%, while the activity of Compound **3** remains almost constant at all concentrations and shows no significant change. This result indicates that the compound does not have the capacity to donate electrons or hydrogen atoms by interacting with the DPPH radical and therefore does not possess any significant antioxidant properties through this mechanism.

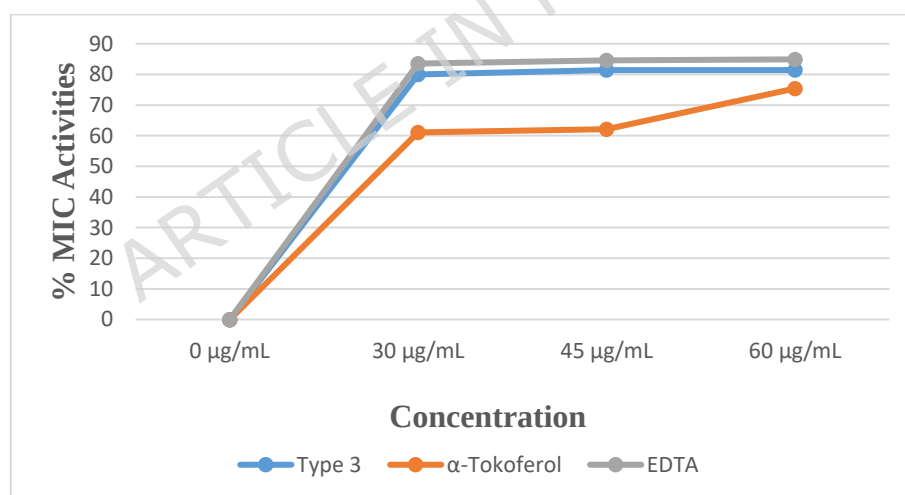


Figure 3. % MIC Activites of Compound **3**

The findings in Figure 3 clearly demonstrate that the metal chelation potential of the Compound **3** is high. In particular, the fact that it reaches levels nearly equal to EDTA (over 80%) at a concentration of 30 μ g/mL indicates that the compound has a strong ion binding capacity. At higher concentrations, although the values show slight fluctuations, they remain stable at around 80%. In contrast, α -tocopherol shows a more limited increase in the same dose range, remaining at around a maximum of 75%. This situation demonstrates that the Compound **3** exhibits chelation activity that is quite close to the reference standard EDTA and significantly superior to α -tocopherol.

The results in Table 1 demonstrate that the triazol-5-one derivative with compound number Compound **3** possesses selective yet significant antimicrobial potential against both Gram-positive and Gram-negative bacteria. Among Gram-positive bacteria, *Bacillus subtilis* (21 mm) showed the highest susceptibility, while *Staphylococcus aureus* also exhibited a strong effect with a zone diameter of 18 mm. In contrast, only moderate inhibition (13 mm) was recorded for *Bacillus cereus*. In the Gram-negative group, *Pseudomonas aeruginosa* (20 mm) and *Klebsiella pneumoniae* (17 mm) showed significant inhibition values, but no activity was observed for *Escherichia coli*. While the reference antibiotic ampicillin showed a much higher effect on all microorganisms (33–37 mm), Compound **3** was found to give similar or even superior results to neomycin (13–17 mm) and streptomycin (10–21 mm) in some species. These findings indicate that the compound may be a promising therapeutic candidate, particularly for species such as *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Table 1. Antimicrobial activity results of Compound **3**

Compound No	Microorganisms and zone diameter values* (mm)					
	Gram positive			Gram negative		
	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Compound 3	18 (+++)	21 (+++)	13 (++)	17 (+++)	(-)	20 (+++)
Ampicillin	37 (+++)	33 (+++)	36 (+++)	35 (+++)	34 (+++)	36 (+++)
Neomycin	13 (++)	17 (+++)	17 (+++)	16 (++)	16 (++)	17 (+++)
Streptomycin	21 (+++)	12 (++)	12 (++)	11 (++)	10 (+)	12 (++)

* In the evaluation according to the inhibition diameter: <5.5 mm negative effect (-); 5.5-10 mm low effect (+); 11-16 mm moderate effect (++); ≥17 mm high effect (+++) (Demirbaş et al., 2004).

The initial in vitro analyses conducted confirmed both the structural validity and the biological functionality potential of the compound, providing a strong preliminary assessment for the study. Following these findings, in ovo applications were performed in the continuation of the experimental process, and the results on embryonic development were examined.

Each of the control, DMSO, and experimental groups contained 40 eggs. All groups were incubated under the same environmental conditions, and in ovo applications were performed simultaneously. At the end of the incubation period, hatching rates were 87.5%, 80% and 0% in the control, DMSO and

experimental groups, respectively. This observed result indicates that the applied substances may cause a potential interruption in the embryo development process.

Table 2. In Ovo Feeding Hatching Number and Hatching Rate (%) Findings

Group	Number of Eggs	Injection Material	Injection Volume (mL)	Hatch Number	Hatching Rate (%)
Control	40	-	-	35	87,5
DMSO		DMSO	0.5	32	80
Experiment		15 mg Compound*		0	0

* 1-(Morpholin-4-yl-methyl)-3-(p-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1H-1,2,4-triazol-5-one

Live hatchlings were obtained only in the Control and DMSO groups, whereas no chicks hatched in the Experimental group (Table 2). This observed result indicates that the applied substances may cause a potential interruption in the embryo development process.

Following the hatchability results shown in Table 2, baseline egg weights were compared across groups to ensure that the absence of hatching in the experimental group was not due to initial mass differences. The statistical evaluation of egg weight homogeneity is presented in Table 3.

These hatchability outcomes were further evaluated through statistical analyses, and the results of the variance assessment are presented in Table 3. Levene test results confirmed homogeneity of variances ($p = 0.385$). One-way ANOVA and Tukey post-hoc analyses indicated no significant differences in egg weights among the groups ($P > 0.05$), confirming that the absence of hatchability in the Experimental group resulted from compound-specific effects rather than initial egg mass.

Table 3. In Ovo Feeding Egg Weight Findings

Group	Number of Eggs	Injection Material	Injection Volume (mL)	Egg Weight $\bar{x} \pm S\bar{x}^{**}$	P
Control	40	-	-	60,29±0,57	0,385
DMSO		DMSO	0.5	60,22±0,46	
Experiment		15 mg Compound*		61,14±0,43	

*1-(Morpholin-4-yl-methyl)-3-(p-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1H-1,2,4-triazol-5-one

**Mean (x) and standard error (Sx) values of animals in each group (n:40).

Discussion

The FT-IR, ^1H -NMR, and ^{13}C -NMR data confirmed that the synthesized compound, 1-(morpholin-4-yl-methyl)-3-(*p*-chlorobenzyl)-4-(4-hydroxybenzylideneamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one, possesses the expected structural features (Supplementary Material, Scheme S1 and Table S2). Detailed evaluation of the spectroscopic findings verified the successful completion of both the Schiff base formation and the Mannich reaction, indicating that the compound was obtained in accordance with the intended chemical structure. These characterization steps were performed to confirm the structural integrity of the molecule prior to in ovo administration and to ensure its reliable use in subsequent biological assays.

The structural confirmation of the triazol-5-one derivative also underscores the importance of subjecting the compound's potential interactions within biological systems to experimental preliminary evaluation, rather than relying solely on theoretical predictions. In this context, the spectroscopic validation constituted a fundamental prerequisite for subsequent experimentation. Before progressing to in ovo application, in vitro antioxidant and antimicrobial assays were conducted to obtain screening-level, preliminary information regarding the compound's interaction potential in biological environments. These preliminary assessments contributed to interpreting the embryonic responses observed during the in ovo evaluation phase within a data-consistent framework and supported the consideration of potential biological interactions that may be associated with hatching success or failure.

The data obtained from the study indicate that the synthesized Compound **3** triazol-5-one derivative exhibits a heterogeneous profile in terms of antioxidant mechanisms. The fact that the compound remains at low levels in reduction power tests at all concentrations and does not show a significant increase compared to standard antioxidants indicates that reduction pathways are not a dominant antioxidant mechanism for this compound. Similarly, the negligible capacity to scavenge the DPPH radical suggests that the compound has a weak electron or hydrogen atom donation property. In contrast, when its metal ion chelation capacity is evaluated, its performance, which is particularly close to that of EDTA, indicates a pronounced interaction potential with transition metal ions involved in free radical-related processes. Antimicrobial findings similarly point to a selective effect profile. High inhibition was observed in the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, while *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in the Gram-negative group showed significant sensitivity. The absence of any effect in *Escherichia coli* reveals that the compound produces different biological responses depending on the bacterial species. Although ampicillin showed stronger activity in all cases, it is noteworthy that the Compound **3** yielded results comparable to neomycin and streptomycin in some bacterial species. These findings provide screening-level insight into the biological activity spectrum of

the compound and may be informative for structure–activity relationship considerations in future studies.

The findings of the study reveal that the Compound **3** triazole-5-one derivative does not possess a strong reduction or radical scavenging effect in its biological activity profile, but it offers a notable interaction potential in metal chelation capacity. When evaluated in terms of antimicrobial activity, the compound's marked inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* indicates a selective antimicrobial response under the tested conditions. Although it exhibits weaker activity compared to standard antibiotics, it is noteworthy that it provides comparable results in certain cases. The results of this study indicate that the compound in question may be considered for further investigation in specific biological contexts, within the scope of preliminary screening data.

The high mortality observed following the in ovo administration of Compound **3** suggests that multiple adverse biological processes may be involved when evaluated together with the compound's defined biological characteristics. In this context, the proposed mechanisms are interpreted as plausible processes associated with the observed findings. Despite its very weak reducing power and limited free radical-scavenging capacity in vitro, its metal-chelation activity was found to be high and stable, approaching that of EDTA. This property may have contributed to the sequestration of essential ions such as Fe^{2+} , Zn^{2+} , and Cu^{2+} within embryonic tissues, thereby potentially affecting metalloenzyme-dependent processes. Together with its limited antioxidant capacity, these potential effects may have compromised embryonic metabolic balance, particularly during late-stage development. In addition, the compound's antimicrobial activity, which is known to involve interactions with cell membranes and metabolic enzymes in microbial systems, may have exerted nonspecific disruptive effects within embryonic cells. Furthermore, the suspension-based administration route may have resulted in localized regions of elevated compound concentration, potentially aggravating tissue exposure. Taken together, these observations suggest that the adverse embryonic outcome associated with Compound **3** may be related to a combination of metal-chelation capacity, limited antioxidant defenses, and nonspecific cellular stress, rather than a single dominant mechanism.

The statistical analysis results support that the observed embryotoxic effect was associated with the compound. Levene's test and ANOVA confirmed that initial egg weights were similar across groups ($p > 0.05$), demonstrating that the absence of hatching in the experimental group was not attributable to initial egg mass. While hatchability was 87.5% in the Control group and 80% in the DMSO group, the complete absence of hatching in the Experimental group suggests that the adverse outcome may be related to the active compound, rather than the solvent or the injection procedure. The loss of all embryos following the injection on day 17 indicates a potential impact on late-stage embryonic development. This developmental window is characterized by increased oxygen demand and accelerated metabolic

activity, which may render embryos more susceptible to mechanisms such as metal chelation, oxidative stress, and mitochondrial dysfunction. Accordingly, when the statistical findings are evaluated together with the *in vitro* biological properties of the compound, the observed embryonic mortality appears consistent with the compound's structural and biochemical profile.

This study contributes to the literature as a pioneering experimental study evaluating the applicability of a triazol-5-one-based compound to the embryo via the *in ovo* method. The failure of hatching to occur at the 510th hour of incubation suggests that, under the tested conditions, this structural class may have induced a high level of physiological stress during embryonic development, thereby indicating the need for careful reassessment of its biological safety profile through further studies.

Conclusion

The structural confirmation of the synthesized triazol-5-one derivative ensured that the compound possessed the required chemical integrity for subsequent biological evaluation. Spectroscopic validation, supported by detailed FT-IR and NMR data (Supplementary Material), provided a reliable basis for *in ovo* experimentation and enabled a more accurate interpretation of embryonic responses observed in this study.

When the *in vitro* biological properties and the *in ovo* embryonic responses to the p-chlorobenzyl-substituted triazol-5-one derivative are evaluated together, the findings suggest that the compound, under the tested dose and formulation, was not tolerated by broiler embryos. The high metal-chelating capacity, low antioxidant activity, and membrane-disruptive antimicrobial characteristics identified *in vitro* are consistent with the 100% mortality observed following *in ovo* administration, indicating an adverse embryonic outcome under the experimental conditions. Statistical analyses confirmed that initial egg weights did not differ among groups, thereby verifying that the observed mortality was not attributable to procedural factors and was associated with exposure to the active compound.

These results demonstrate that safe dose ranges of triazol-5-one derivatives in avian embryonic systems have not yet been clearly defined under *in ovo* conditions, and that late-stage embryonic development may be particularly sensitive to mechanisms such as metal chelation, oxidative stress, and mitochondrial dysfunction. Nevertheless, this study provides initial experimental evidence regarding the *in ovo* applicability of triazole derivatives, offering a scientific foundation for future investigations evaluating lower or fractionated doses, alternative solubilization strategies, or structurally distinct triazole analogs. Clarifying the embryonic safety profile of such compounds under controlled experimental conditions is essential for determining their potential pharmacological or antibacterial applications.

This finding also underscores the inherent limitations of administering pharmacological agents within *in ovo* systems and highlights important methodological constraints of the approach. Although the planned biochemical and physiological measurements could not be performed due to the absence of

hatching, should not be interpreted as diminishing the scientific value of the study; rather, it provides important preliminary insight regarding the feasibility and boundaries of pharmacological compound administration during embryogenesis. The present experiment contributes early empirical data evaluating triazol-5-one derivatives in an in ovo context. Future studies incorporating alternative derivatives, lower or fractionated doses, and optimized solubilization strategies will be essential for clarifying the potential roles and limitations of this compound class in poultry production systems.

This study has certain limitations, including the evaluation of a single dose and formulation, the inability to perform post-hatch biochemical and physiological analyses due to complete embryonic mortality, and the inherent constraints of the in ovo model. Accordingly, the findings should be interpreted within the scope of the experimental conditions applied.

Declarations

Acknowledgements

The authors would like to acknowledge Dr. Muzaffer Alkan for reporting the Schiff base compound used at the initial stage of the synthesis in his doctoral dissertation. In addition, we would like to thank Yavuz Yadigar, Executive Director of Production Operations at Banvit Co., for the supply of fertilized eggs.

Funding

This study was supported by the Scientific Research Projects Coordination Unit of Dicle University (DÜBAP) under the Open Access Publication Support Project (Project No: VETERINER.25.030).

Consent for publication

Not applicable.

Ethical Statement

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAUHADYEK /2019-086)

Authors' contributions

OD, SUB, SB, GY and HY executed the experiment; OD and SUB completed the manuscript; OD, SUB and SB were in charge of laboratory analyses. OD was responsible for supervision and writing of the manuscript. All authors reviewed the manuscript. In addition, the Mannich base derivative (Compound 3) used in this study was previously reported in the doctoral dissertation of SB.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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