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Enhancing Antioxidant and Biochemical Markers of Broilers *via In Ovo* Injection with Peppermint Oil

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Abstract: This study was designed to investigate the influence of *in ovo* injection of peppermint (*Mentha piperita*) essential oil (PO) on hatchability (%), antioxidant states, and some biochemical parameters of broilers at hatch. Fertilized eggs (120) were divided into six groups, the control group and other groups injected with 10, 50, 100 and 150 µL/ml of PO in saline solution on the 18th day of incubation in an air sac. GC-MS indicated that PO contains L-menthone (32.7%), menthol (29.34%) and pulegone (9.63 %) as major components. The results revealed that injection of 10 µL/mL of PO increased the hatchability compared to other groups while the body weight of the chicks was not significantly different. Antioxidant activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) significantly increased in injected groups compared to the control group. In addition, plasma total protein and globulin levels increased while the albumin/globulin (A/G) ratio was reduced. Lipid profile indicated that plasma cholesterol and triglyceride levels were significantly decreased in all PO-treated groups. In conclusion, *in ovo* injection with 10 µL/mL of PO showed positive effects on hatchability (%) and the viability of post-hatch chicks, without indicated harm to the blood constituents.

1 Introduction

The poultry industry faces significant challenges, including oxidative stress, which adversely impacts production and chick quality. In broiler embryos, free radicals and reactive oxygen species (ROS) are produced by rapid growth and metabolisms of broiler embryos, which can harm tissues rich in polyunsaturated fatty acids by targeting their carbon-carbon double bonds and exacerbating oxidative stress. Prolonged hatch windows and delayed chick collection from incubators intensify this problem, overwhelming antioxidant defense systems, impairing immune responses and increasing corticosterone levels (Deeming and Pike 2013, Arab-Ameri et al 2016, Oke et al 2021).

To mitigate oxidative stress in broiler embryos, antioxidant interventions particularly through *in ovo* injection have been proposed. This technique delivers essential nutrients directly to the developing embryo, enhancing antioxidant defenses and reducing oxidative damage (Uni and Ferket 2004, Oke et al 2024). Among antioxidants, essential oils (EOs) have gained interest for their availability and potent bioactive properties.

For example, Sulaiman and Tayeb (2021) demonstrated that eggs injected with rosemary oil in broilers improved hatchability, growth performance, feed intake, feed conversion ratios and immune responses. Peppermint essential oil (PO) has shown antioxidant, antifungal, antiviral, antibacterial and anti-inflammatory properties (Bupesh et al 2007, Nickavar et al 2008, Carretto et al 2011, Sun et al 2014, Brand et al 2016). Dietary supplementation with PO has been linked to enhanced performance during early life stages (Toghyani et al 2010). Further research indicates that peppermint leaves and menthol improve broiler growth (Abdel-Wareth et al 2019), reduce abdominal fat (Ocak et al 2008, Khempaka et al 2013) and enhance immunity while lowering oxidative stress (Al-Kassie 2010, Arab-Ameri et al 2016).

Despite these findings, the effects of *in ovo* injection with PO on broiler chicks have not been explored. The present study aims to reduce oxidative stress, increase hatchability, and enhance immune responses through *in ovo* injection with PO. Specifically, the study evaluates its effects on hatchability rates, lipid profiles, protein profiles and antioxidant status in broiler chicks. By addressing these outcomes, this research highlights the PO potential as a natural intervention to improve broiler chick viability and performance.

2 Materials and Methods

2.1 Extraction of PO

PO was extracted by hydrodistillation (Desam et al 2019) from the dry aerial parts of *Mentha piperita*.

2.2 GC-MS analysis

PO was analyzed by using a Shimadzu GCMS-QP2020 system (Tokyo, Japan). An Rtx-1MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) from Restek and a split-splitless injector were installed in the system. The column temperature was 45°C, held for 2 minutes, followed by a temperature program that included increasing temperature to 300°C at a rate of 5°C/min then held for 5 minutes. The injector temperature was set to 250°C; helium was used as the carrier gas with a flow rate of 1.41 mL/min. For mass spectrometry, the filament emission current was set to 60 mA, the ionization voltage was 70 eV, and the ion source temperature was 200°C. A diluted sample (1% v/v) was injected in split mode with a split ratio of 1:15, and the mass spectra were recorded under these conditions.

2.3 Experimental design

A total of 120 fertilized eggs from Ross 308 broiler breeders (average weight 50 g) were split up into six groups to study the effects of *in ovo* PO injection. The first group served as untreated control, while the remaining five groups were injected with 0.1 mL of physiological saline (0.9% NaCl) containing peppermint oil at concentrations of 10, 50, 100 and 150 µL/mL. The oil was injected into the air sac on the 18th day of incubation under sanitized conditions.

2.4 Blood biochemical analysis

Samples were collected at hatch (day-old chick) from each treatment *via* the jugular vein. The samples were centrifuged at 4000 rpm for 15 minutes; the resulting plasma was stored at -20°C until analysis. EDTA was used as a blood anticoagulant. Biochemical measurements include malondialdehyde (MDA) content (Kei 1978), enzyme activities i.e. SOD (Marklund and Marklund 1974), CAT (Aebi 1984) and GPx (Paglia and Valentine 1967), lipid profile parameters i.e. total cholesterol (T. chol), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (Friedewald et al 1972, Bucolo and David 1973, Meiattini et al 1978, Grove 1979) and protein profile parameters i.e. total protein, albumin and globulin (Doumas et al 1981, Busher 1990, Doumas et al 1997). Measurements were conducted using commercial kits from the Egyptian Company for Biotechnology (Obour City, Cairo, Egypt).

2.5 Statistical analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) following the General Linear Model (GLM) procedure in SAS (SAS User's Guide, 2006). Differences among means were determined using Duncan's multiple-range test (Duncan, 1955).

3 Results and Discussion

3.1 Chemical composition of PO

Table 1 and **Fig 1** showcase the chemical composition and GC-MS chromatogram of PO. The GC-MS analysis identified 20 volatile compounds (based on the Mass Spectrum and Retention Index), with the major components being L-menthone (32.70%), menthol (29.34%) and pulegone (9.63%). Minor constituents included menthyl acetate (5.72%), iso-menthone (4.34%), neomenthol (4.34%), 1,8-cineole (3.23%) and piperine (1.97%), alongside smaller quantities of com pounds such as caryophyllene, menthofuran, and spathulenol. The mass spectra of the major constituents are illustrated in **Fig 2**. These compounds are primarily responsible for PO's antioxidant, anti-inflammatory, and antimicrobial properties. The results align with Wu et al (2019), and Zhang et al (2023), who identified menthone and menthol as the key components of peppermint oil. However, differences in chemical composition observed by researchers like Floare et al (2023) and Yadegarinia et al (2006) underscore the influence of factors e.g. harvest season, climate, soil type, and geographic location, as emphasized by Hudz et al (2023).

Table 1. Chemical composition of Peppermint (*Mentha piperita*) essential oil (PO)

No	Name of compounds	Retention time (min)	Area%	Mass
1	α-Thujene	7.598	0.46	136.1252
2	Phellandrene	8.758	0.48	136.1252
3	1,8-Cineole	10.288	3.23	154.13577
4	**L -Menthone**	**13.863**	**32.7**	**154.13577**
5	Isomenthone	14.082	4.34	154.13577
6	Menthofuran	14.256	1.44	150.10447
7	Neomenthol	14.378	4.34	156.15142
8	**Menthol**	**14.654**	**29.34**	**156.15142**
9	Neoisomenthol	14.907	0.43	156.15142
10	Terpinol	15.043	0.41	154.13577
11	**Pulegone**	**16.268**	**9.63**	**152.12012**
12	Piperitone	16.602	1.97	152.12012
13	Neomenthylacetate	17.687	0.76	198.16198
14	Methyl acetate	18.182	5.72	198.16198
15	Germacrene	21.193	0.35	204.1878
16	Caryophyllene	21.925	1.62	204.1878
17	Spathulenol	25.649	0.28	220.18272
18	Caryophllene oxide	25.780	0.5	220.18272
19	Glubulol	26.075	0.39	222.19837
20	Mint sulfide	29.363	0.44	236.15987

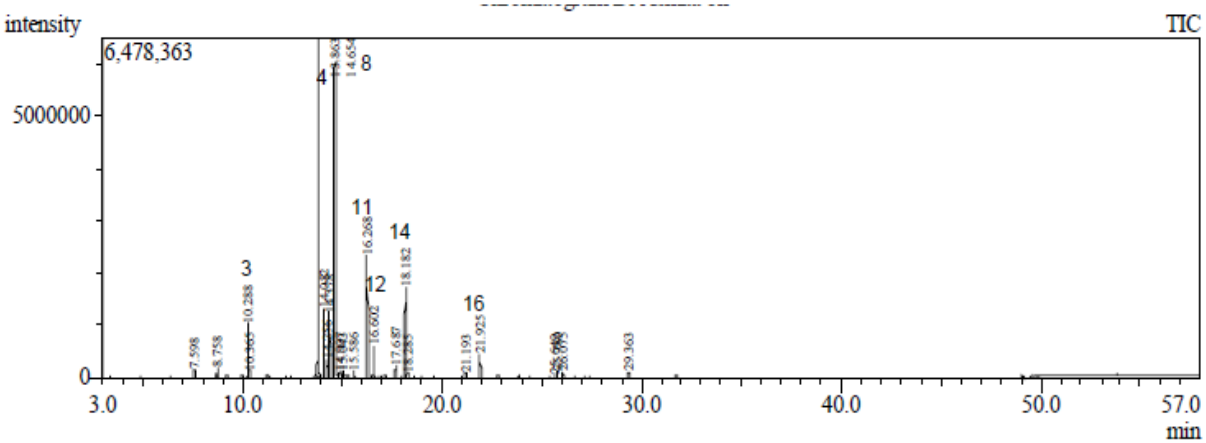

Fig 1. GC Chromatogram of peppermint oil

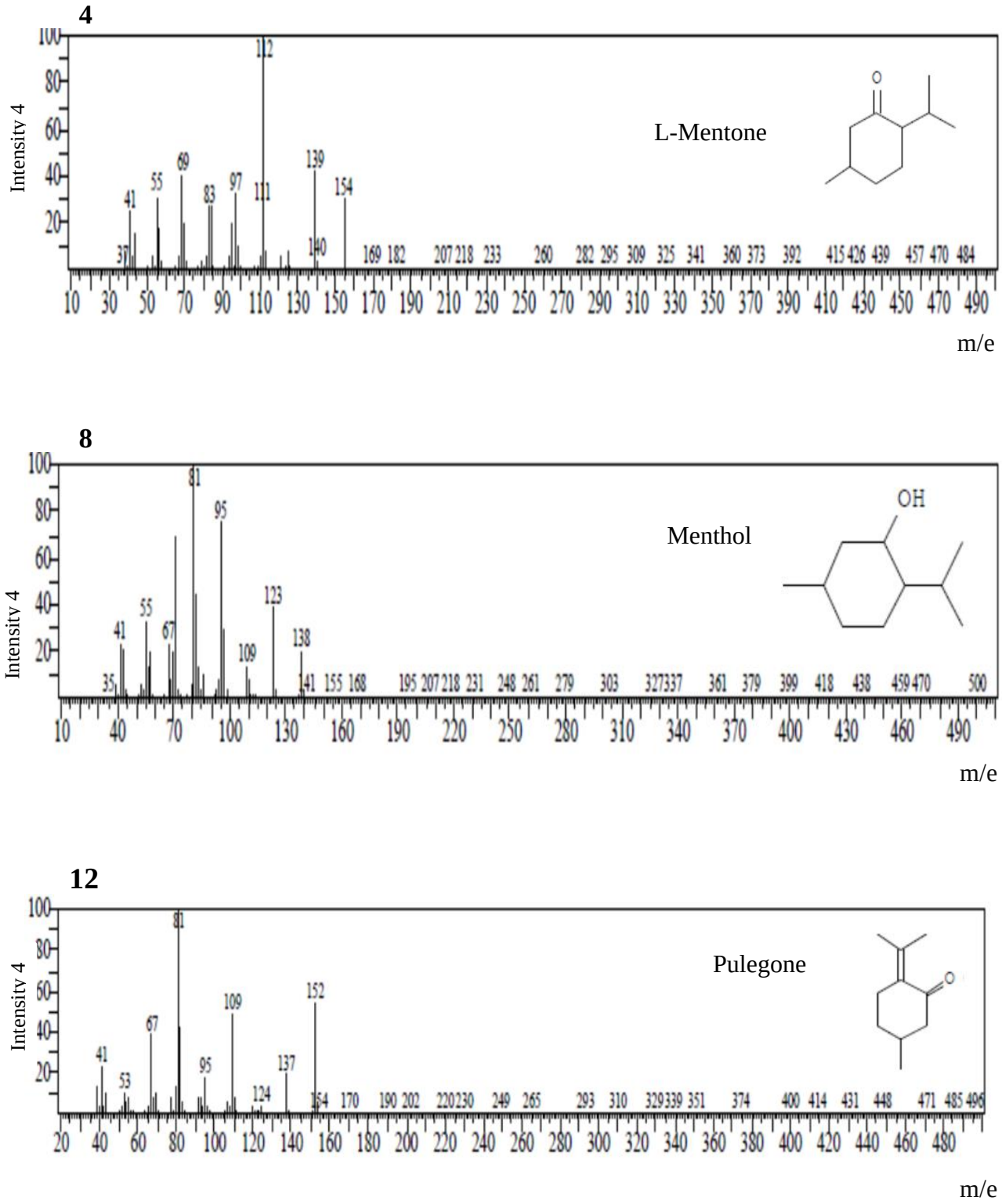

Fig 2. Mass spectra of major constituents of peppermint oil: 4: mass spectrum of L-mentone 8: mass spectrum of menthol, 11: mass spectrum of pulegone

3.2 Impact on hatchability (%) and chick body weight

Fig 3 illustrates the influence of PO injection on egg hatchability (%). Hatchability was highest (80%) in group 3 (injected with 0.1 mL of PO at 10 µL/mL) compared to the control group (75%). However, hatchability declined significantly at higher doses (100 and 150 µL/mL) to 65% and 55%, respectively. These findings are consistent with other studies; for example, Aberbour et al (2023) reported increased hatchability with low-dose rosemary oil in Japanese quail, and Gupta et al (2022) found decreased hatchability at higher doses of α-ketoglutaric acid in broilers.

The positive effect of low-dose PO on hatchability could be attributable to the antioxidant activity of its constituents, which enhances scavenging free radicals, supporting embryonic development. Conversely, lower hatchability in groups 5 and 6 (higher doses) could result from the toxic effects of PO constituents like menthol, pulegone, and menthofuran, which are known for their hepatotoxicity and neurotoxicity at high concentrations (Balakrishnan 2015). Similar results were published by Zhang et al (2021) for low-dose EOs particularly for individual components such as thymol and carvacrol, while Sulaiman and Tayeb (2020) observed increased hatchability with rosemary, almond and olive oils.

Regarding chick body weight, data in **Table 2** showed no significant differences among treatments, indicating that PO injection had no adverse effects on embryonic development. These findings align with Oke et al (2021), who observed no impact on chick weight with black cumin extract, and Toosi et al (2016), who reported no significant effects with essential oil and organic acid blends. However, El-Kholy et al (2021) found increased body weight with cinnamon, thyme and clove extracts. In conclusion, injection with a low dose of PO within eggs positively influences hatchability (%) without detrimental effects on body weight in day-old chicks.

3.3 Effects on lipid profile

Table 3 shows that *in ovo* injection with PO significantly reduced T. G, T. chol, LD, and VLDL levels in comparison to the control (group 1) and saline-injected group (group 2) at hatch. However, HDL-cholesterol level was slightly increased. These findings align with El-Speiy et al (2020) and Aberbour et al (2023), who demonstrated that PO can inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase which controls cholesterol synthesis in the liver. Similarly, Sulaiman and Tayeb (2021) observed significant reductions in T.chol and T.G levels in broilers following rosemary oil injection within eggs. In contrast, Oladokun et al (2021) reported no effect on total cholesterol levels with an injection blend of essential oils; Gupta et al (2022) found also no changes in cholesterol levels in newly hatched chicks injected with α-ketoglutaric acid. The results of the present study highlight PO's lipid-lowering potential through modulation of cholesterol synthesis and lipid metabolism, besides the slight increase in HDl-cholesterol level.

3.4 Influence on protein profile

Table 4 demonstrates that there is a significant increase in plasma total protein and globulin levels, along with a decrease in the albumin-to-globulin (A/G) ratio in broilers subjected to the PO *in ovo* injection while albumin levels were not significantly affected. Similar results were observed with Ahmed et al (2016), who mentioned that supplementation of broiler diets with PO significantly elevated plasma total proteins and globulin without altering albumin levels. Similarly, Sulaiman and Tayeb (2020) observed an increase in total protein and globulin levels *via* injection of rosemary oil. In contrast, Gupta et al (2022) found no changes in plasma proteins in newly hatched chicks injected with α-ketoglutaric acid, suggesting that total protein levels may increase with age, as observed at 21 days of development. This age-related effect was also emphasized by Oke et al (2021), who noted no differences in total proteins, albumin, or globulin levels in day-old chicks injected with black cumin extract. These findings indicate that PO positively affects protein metabolism at hatch, although further studies at later developmental stages may provide deeper insights.

3.5 Impact on lipid peroxidation

Malondialdehyde (MDA) was determined as a biomarker of lipid peroxidation and oxidative stress. **Table 5** shows that PO *in ovo* injection at concentrations of 10, 50, 100, and 150 µL/mL significantly decreased MDA levels in newly hatched chicks compared to control and saline-injected groups. During the final stages of incubation, the increased oxygen consumption results in higher levels of ROS, which oxidizes yolk lipids and elevates MDA concentrations. The reduction in MDA levels observed in the present study can be

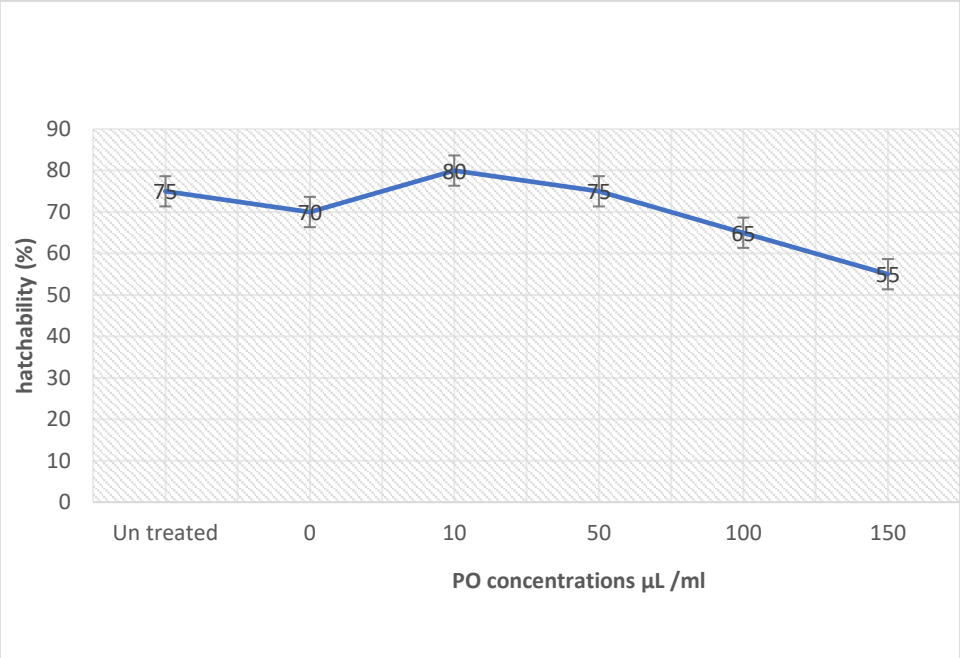

Fig 3. Influence of *in ovo* injection with different concentrations of peppermint oil on the percentage of hatchability

Table 2. Effect on chick body weight at hatch

	G1	G2	G3	G4	G5	G6
Chick weight at hatch (g)	40.16±2.58 ^{a}	37.49± 1.30 ^{a}	39.66 ± 0.60 ^{a}	39.8 ±1.27 ^{a}	40.16 ±1.96 ^{a}	38.86 ±2.3 ^{a}

G1: control group (untreated), G2: injected with 0.1 mL of physiological saline (0.9% NaCl), Groups 3, 4, 5, and 6 were injected with 0.1 ml PO at 10, 50, 100, and 150 µL/mL, respectively. Different letters within rows refer to significant differences (P< 0.05).

Table 3. Plasma lipid profile of hatched broiler chicks following *in ovo* injection with different levels of peppermint oil

	G1	G2	G3	G4	G5	G6
Total cholesterol (mg/dl)	192.66±2.027 ^{a}	192.33 ±2.603 ^{a}	163.33 ±2.33 ^{b}	163.66±3.527 ^{b}	167.33±2.905 ^{b}	164.33 ±3.28 ^{b}
Triglyceride (mg/dl)	160 ±1.73 ^{a}	161 ±1.73 ^{a}	111.66 ±5.811 ^{b}	110 ±4.041 ^{b}	109.33±4.33 ^{b}	116.33±4.807 ^{b}
HDL (mg/dl)	62.33 ±2.02 ^{b}	68.33±1.33 ^{b}	75 ±2.64 ^{a}	73.66 ±2.905 ^{a}	78.66 ±1.201 ^{a}	75.66 ±2.027 ^{a}
LDL (mg/dl)	98.33 ±0.17 ^{a}	91.8±4.0 ^{a}	66 ±3.06 ^{b}	68 ±1.70 ^{b}	66.8 ±2.457 ^{b}	65.4 ±2.11 ^{b}
VLDL (mg/dl)	32 ±0.346 ^{a}	32.2 ±0.346 ^{a}	22.33 ±1.162 ^{b}	22 ±0.808 ^{b}	21.86 ±0.866 ^{b}	23.26 ±0.961 ^{b}

G1: control group (untreated), G2: injected with 0.1 mL of physiological saline (0.9% NaCl), Groups 3, 4, 5, and 6 were injected with 0.1 ml PO at 10, 50, 100, and 150 µL/mL, respectively. Different letters within rows refer to significant differences (P< 0.05).

Table 4. Plasma protein profile of broiler chicks at hatch following *in-ovo* injection with different levels of peppermint oil

	G1	G2	G3	G4	G5	G6
Total Protein (g/dl)	2.86 0.181 ^{b}	2.87 ±0.114 ^{b}	4.233±0.240 ^{a}	4.566±0.392 ^{a}	4.253 ±0.157 ^{a}	4.433 ±0.433 ^{a}
Albumin (g/dl)	1.45 ±0.103 ^{a}	1.46 ±0.07 ^{a}	1.526 ±0.037 ^{a}	1.523 ±0.070 ^{a}	1.403±0.153 ^{a}	1.556 ±0.037 ^{a}
Globulin (g/dl)	1.403 ±0.079 ^{b}	1.413±0.046 ^{b}	2.706 ±0.270 ^{a}	3.043 ±0.322 ^{a}	2.85 ±0.252 ^{a}	2.87 ± 0.415 ^{a}
A/G	1.033±0.027 ^{a}	1.026±0.02 ^{a}	0.576 ±0.066 ^{b}	0.503 ± 0.033 ^{b}	0.480 ±0.075 ^{b}	0.560 ± 0.07 ^{b}

G1: control group (untreated), G2: injected with 0.1 mL of physiological saline (0.9% NaCl), Groups 3, 4, 5, and 6 were injected with 0.1 ml PO at 10, 50, 100, and 150 µL/mL, respectively. Different letters within rows refer to significant differences (P< 0.05).

Table 5. MDA (nmol/ml) of hatched broiler chicks following *in ovo* injection with different levels of peppermint oil

	G1	G2	G3	G4	G5	G6
MDA (nmol/m)	0.652 ± 0.044 ^{a}	0.619 ±0.068 ^{a}	0.229 ±0.054 ^{b}	0.262 ±0.042 ^{b}	0.25 ±0.068 ^{b}	0.378 ±0.065 ^{b}

G1: control group (untreated), G2: injected with 0.1 mL of physiological saline (0.9% NaCl), Groups 3, 4, 5, and 6 were injected with 0.1 ml PO at 10, 50,100, and 150 µL/mL, respectively. Different letters within rows refer to significant differences (P< 0.05).

linked to increased activity of antioxidant enzymes due to PO *in ovo* injection. These results align with Aguiar et al (2023), who reported decreased MDA levels with PO dietary supplementation in juvenile Nile tilapia. Similarly, El-Speiy et al (2020) noted that supplementing rabbit diets with PO (800 mg/kg) during the summer significantly reduced serum MDA levels. These results highlight the potential of PO *in ovo* injection to mitigate oxidative damage through its antioxidant properties.

3.6 Influence on antioxidant defensive enzymes

Table 6 demonstrates that *in ovo* injection with PO at different concentrations (10, 50, 100, 150 L/ml) significantly increased SOD, CAT and GPx, in newly hatched chicks. This enhancement can be attributed to the antioxidant properties of PO providing better protection against free radicals that can harm embryonic development. These findings are consistent with Oke et al (2021), who reported that black cumin extract injection in eggs reduced plasma MDA levels and increased SOD activity, emphasizing the importance of antioxidants in chick development.

The antioxidant property of PO is likely linked to its major ingredient, menthol, as reported by Ramos et al (2017), who associated PO's radical scavenging activity with the hydroxyl radical. Bastaki et al (2018) further established that menthol mitigated oxidative stress in rat colonic mucosa. Similarly, Aly et al (2023) observed that L-menthol supplementation enhanced total antioxidant capacity and GPx activity in Japanese quail.

However, some studies suggest that the antioxidant properties of EOs are not attributable to their major constituents. Dhifi et al (2016) highlighted that minor compounds, such as certain alcohols, ethers, ketones and monoterpenes like 1,8-cineole and isomenthone, contribute significantly to the overall activity and may have synergistic effects. de Sousa et al (2023) emphasized that both major and minor compounds of EOs play crucial roles in their biological properties. These findings suggest that the observed increase in antioxidant enzyme activity in this study could result from the combined effects of menthol and other minor components in PO.

Table 6. Antioxidant defensive enzymes of broiler chicks at hatch following *in ovo* injection with different levels of peppermint oil

	G1	G2	G3	G4	G5	G6
GPx (nmol/ml)	1.134 ±0.176 ^{b}	1.034 ± 0.141 ^{b}	2.098 ± 0.214 ^{a}	2.238 ±0.230 ^{a}	2.181 ±0.492 ^{a}	2.627 ± 0.3003 ^{a}
SOD U/ml	2.64 ±0.156 ^{b}	2.64 ± 0.059 ^{b}	3.38 ± 0.141 ^{a}	3.72 ±0.248 ^{a}	3.72±0.265 ^{a}	3.69 ±0.366 ^{a}
CAT nmol/ml	0.76 ±0.058 ^{b}	0.85±0.088 ^{b}	1.41±0.086 ^{a}	1.40 ±0.18 ^{a}	1.60 ±0.077 ^{a}	1.47 ±0.089 ^{a}

G1: control group (untreated), G2: injected with 0.1 mL of physiological saline (0.9% NaCl), Groups 3,4,5,6 were injected with 0.1 ml PO at 10, 50,100,150 µL/mL, respectively. Different letters within rows refer to significant differences (P< 0.05).

4 Conclusions

In ovo injection of 10 µL/mL peppermint oil (*Mentha piperita*) was found to have a positive effect on hatchability and post-hatch chick viability in addition to reducing oxidative stress in chicks, without adversely affecting the blood constituents of broilers. The results suggest that peppermint oil can be used as an effective antioxidant to mitigate oxidative stress during embryonic development through *in ovo* injection. Additionally, further research is recommended to explore the antioxidant effects of peppermint oil on broiler chicks, as it may offer valuable benefits for enhancing chick health and development in the later stages.

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