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Competitive Study Between Raw and Nano-Propolis as Feed Additive in Broiler and its Effect on the Immune System

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Abstract | Propolis is one of the natural bee products with many biological properties. It is well known by enhancing digestion and nutrient absorption, immunostimulant and hepatoprotective effect. Nanopropolis is a natural nanomaterial that could be used to improve the properties of propolis to be easily applied. Propolis nanoparticles are easily absorbed and have better antibacterial and antifungal effects than ordinary propolis. The present study aimed to compare propolis ethanolic extract (PEE) and propolis nanoemulsion (PN) as a feed additive in poultry farms by evaluating their influences on growth, immune status, and pathological conditions in chickens. The size, uniformity, and spherical shape of PN were measured at 4.065 ±1.12nm, with a polydispersity index of 0.021 which allows for easy blood vessel passage and renal clearance, fresh preparation without adding stabilizer cause zeta potential was recorded at -10 mV. Half maximal inhibitory concentration (IC_{50}) of PN efficacy was found to be 111.5 ug/mL and had a functioning group-specific fingerprint that showing distinct bonds formed by the conversion of the pure propolis100% to nano-propolis 20% compound . The obtained data illustrated a significant impact on feed intake in the (PN) group and the feed conversion ratio in the (PN) and (PEE) groups compared to control group. PN results in an earlier elevation in lysozyme concentration at 14 days old, while PEE causes late elevation at 28 days old. Both PEE and PN had an equal elevating effect on total antioxidant capacity (TAC) at 14 and 35 days old compared to control group. (PN) group possessed a significant increase in IFN-γ and IL2 more than (PEE) group. Neither PEE nor PN had an effect on HI antibody titer against NDVV. Pathological examination revealed significant alleviation in the immune organ structure of both PEE and PN groups in which lymphoid depletion and inflammatory changes decreased. In contrast, the intestine of the PEE group showed more significant improvement than PN group on 21and28 days old with intact villi and few lymphocytic infiltrations. Indeed, using of PN as an additive with vaccine application in chickens will be of add value in term of growth, immune status and organs health.

Keywords: Propolis, Nanoemulsion, Growth performance, Immunity, Pathological finding

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INTRODUCTION

Propolis (bee glue), a resinous balsamic sticky material, is produced by bees by combining plant resins, saliva, wax, and pollen (Vieira et al., 2022). It is used to preserve the interior of the hives by closing the cracks, fixing the frames, disinfection of honeycomb cells, and mummification of the pests which are killed inside the hives (Kabakci, 2022; El-Sakhawy *et al*., 2023).

The chemical composition of propolis includes various bioactive substances such as polyphenols, steroids, terpenoids, amino acids, vitamins, several micro and macro minerals, and some enzymes (dehydrogenase, glucose-6-phosphatase, acid phosphatase, and adenosine triphosphate (Mona *et al*., 2021). It could be used as a feed supplement due to its high content of minerals, flavonoids, antioxidants, digestive enzymes, and antibacterial substances (Al-Kahtani *et al*., 2022). Also, it has the ability to enhance lipid absorption and liver morphology, lower reactive oxygen species (ROS) generation, and alleviate stress (Dosoky *et al*., 2022), so It could be used as an antioxidant. It was proved by Alkhalefa *et al*. (2022) that the presence of flavonoids, caffeic acid, and esters of aromatic acids in propolis gives it an antiviral effect as they prevent virus transmission to other cells and inhibits virus propagation. Propolis has an antibacterial effect by preventing bacterial DNA replication (Saadawi *et al*., 2022). Kabakci (2022) stated that propolis could be used as a natural alternative in the poultry sector to enhance immunity, reduce oxidative stress, improve feed efficiency, and egg quality. Kuropatnicki *et al*. (2013); Zulhendri *et al*. (2022); Hermansyah *et al*. (2022) had revealed the therapeutic properties of propolis to its content of plant secondary metabolites such as phenolics and terpenoids. The immunomodulatory action of propolis is manifested by improving immune system development and stimulating the activity of T and B lymphocytes, increasing macrophage phagocytic activity and production of interleukins (IL1, IL2, and IL4) and interferon-γ (Sohail, 2017).

Propolis showed a protective outcome against the hazards of various hepatotoxic factors. These factors often lead to degeneration, vacuolar degeneration, steatosis, and necrosis of the liver parenchyma (Klarić, 2014). Abdel-Maksoud *et al*. (2023), reported an improvement in the histologic architecture of lymphoid organs in vaccinated broiler chicken supplemented with propolis. Nanoparticles (NPs) can interact with cellular and molecular components of the immune system. It can bind to certain receptors that are expressed on macrophages surface resulting in bone marrow activation, monocytes mobilization and boosts to microvascular permeability. Also, Neutrophil membrane-coated nanoparticles know how to escape from immune surveillance, neutralize pathological molecules and target inflamed tissue (Liu *et al*., 2022). Level of cytokines were improved by NPs via targeting their source cells and moderating their producing patterns (Ozcan *et al*., 2020; Wu *et al*., 2020).

Nanopropolis refers to propolis that has been processed into nanoparticles, ranging from 1 to 100 nanometers in size. This transformation enhances its absorption and efficacy in the body. The hydrophobic nature of propolis makes it poorly absorbed when consumed in its natural form. By converting propolis into nanopropolis, its bioavailability is significantly improved, making it more effective for medical and biological applications. This process often involves green synthesis methods, which are environmentally friendly and economically feasible. Nanopropolis has shown potential in treating bacterial and fungal infections, and it is also being explored for its applications in food safety and preservation (Hamdi *et al*., 2019; Barsola and Kumari, 2022; Bezerra *et al*., 2023). Adding nanopropolis to the drinking water of broilers can significantly increase overall body weight, weight gain, and feed conversion ratio.

In terms of health, performance, and ongoing feed production, the natural nanomaterial nano-propolis has the potential to benefit veterinary care. Nanoparticles are more easily absorbed by the body, yet nano propolis has stronger antibacterial effects than propolis (Sahlan *et al*., 2017).

Furthermore, Nanopropolis supplementation has been found to increase the activity of antioxidant enzymes like glutathione peroxidase (GPx) and superoxide dismutase (SOD), while reducing malondialdehyde (MDA) levels, which is a marker of oxidative stress (Khalati and Al-Salhie, 2023). Dietary inclusion of nanopropolis liposomes in heat stressed poultry results in better growth indices, feed utilization, and immune function (Eldiasty *et al*., 2024). The interaction of many nanoparticles with immune system is still unclear. So, the immunomodulatory influence of nano propolis needs further clinical studies and long-term safety evaluation. Therefore, this study aimed to evaluate the effect of propolis either as ethanolic extract or as nanoemulsion in modulating the performance and immunity of poultry.

MATERIALS AND METHODS

Animal Ethics

The Institutional Animal Care and Use Committee (ARC-IACUC) at the Agricultural Research Center has approved the protocol (ARC-AHRI-724) in accordance with Article 45 of the 2014 Egyptian Constitution, official decrees of the Ministry of Agriculture No. 27, 1967, the World Organization for Animal Health (WOAH), and the Guide for the Care and Use of Laboratory Animals 8th Edition 2011 (the Guide). After reviewing the ethical consideration of animal use and Animal Welfare that enforces good quality science and the human treatment of animals.

As environmental enrichment we keeping chicks in their housing with other chicks, allowing them to see each other, and providing plenty of bedding. The humane euthanasia we Exsanguination, which involves severing the jugular vein and carotid artery and inserting a knife into the thoracic cavity.

Preparation of Propolis Extract

Ten grams of propolis from Gemiza station of the Agricultural Research Centre were dissolved in 70% ethanol purchased from Loba Co. in a 100 mL volumetric flask at room temperature. The mixture was incubated on a shaker incubator for 5 hours at 25 °C while being vigorously shaken. The resulting supernatant was then transferred to a new container after cooling to room temperature and centrifuging at 1500 rpm for 5 minutes (Saad *et al*., 2024).

Preparation and Characterisation of 20% Propolis Nanoemulsion

Nanoemulsion were created at the Nanotechnology Research Unit. propolis (20 mL), tween 80 (20 mL), and distilled deionized water (60 mL) were blended for thirty minutes using a 1500-watt homogenizer submerged in ice water to avoid producing heat that would have an impact on the active components while they were being prepared. Using a zeta size device, measurements are taken every ten minutes to track the size of the particle. Distilled water was slowly added to the oil phase mixture following the method described by Saad *et al*. (2024).

To ascertain sample size-dependent heterogeneity, distinguish between nanoemulsion and assess the electrical conductivity, zeta potential, droplet size, and polydispersity indices (PDI). The Microtrac FLEX (12.0.1.0) instrument may experience polydispersity of nanoemulsion as a result of sample size dispersion, accumulation, or aggregation during separation or analysis.

Dynamic Light Scattering (DLS) is a well-established and precise measuring technique for characterizing particle sizes in suspensions and emulsions. Microtrac is a pioneer in particle analysis technology, having developed optical systems based on Dynamic Light Scattering for more than 30 years. Microtrac's NANOTRAC Wave II / Zeta is a highly adaptable Dynamic Light Scattering (DLS) analyzer that measures particle size, zeta potential, concentration, and molecular weight. It enables faster measurements using trustworthy technology, more precision, and greater accuracy. All of this has resulted in a tiny DLS analyzer with a new fixed optical probe.

The NANOTRAC Wave II / Zeta's innovative and versatile probe design, combined with the use of the Laser Amplified Detection technique, allows the user to select from a wide range of measuring cells that meet the needs of all applications. This design also enables for measurements of samples with a wide concentration range, as well as monomodal or multimodal samples, without knowing the particle size distribution beforehand.

At Cairo University's Faculty of Agriculture, high-resolution transmission electron microscopy (HRTEM) was used to investigate the electrical conductivity and characteristics of the nanoemulsion. A JEM 1400F HRTEM with a 300 keV beam energy was used for this purpose.

Electron beams serve as the light source, with a possible resolution of sub-nanometers. As a result, the very fine details of nanomaterials become amplified.Transmission electron microscopy (TEM) uses a high voltage electron beam generated by a cathode and focussed by a lens. Initially, the sample is placed in a vacuum. The high voltage electron beam initially passes through the sample before concentrating and amplifying the transmitted electrons. The beam's impact on a phosphor screen, photographic plate, or other light-sensitive sensor generates a picture. TEM only produces a pair of two-dimensional images of the sample; however, these images and diffraction patterns provide information on the internal structure of the materials. (Kannan, 2018).

Thermo Nicolet 380 Fourier Transform Infrared (FT-IR) spectrometer, which is fitted with the Smart SplitPea horizontal Attenuated Total Reflectance (ATR) microsampling device, was used to examine the chemical signature of the nanoemulsion. Triton 10x objective with 0.71 numerical aperture is fitted with Thermo Electron Nicolet Centaurus FT-IR Contact Alert microscope. The Omnic PC workstation is used to control the system.

Mtt Cytotoxicity Assay

Vero cell line (Green monkey cell line) purchasing from VACSERA CO. (Doki, Giza, Egypt) is used for cytotoxicity for determined the safety dose. The cells were cultivated at 37 °C in a humidified atmosphere containing 5% (v/v) CO2. 10% heat-inactivated fetal bovine serum, 100 mg/ml streptomycin, and 100 units/ml penicillin were added to the DMEM culture medium. To assess cell viability at dosages of zero (control) 0, 6.25, 12.5, 25, 50 and 100 ug/ml with three replicated, MTT assay using dimethyl thiazolyl diphenyl tetrazolium salt, a type of tetrazole. Experiment was performed as part of the cytotoxicity test.

Stock solutions of the medication were made with 10% DMSO in ddH2O. Further dilutions were made using DMEM to create working solutions for determining the IC_{50}. The cytotoxic activity of the material was detected in BNL cells using the MTT method with slight adjustments. The cytotoxicity percentage compared to untreated cells was calculated using the following equation. The cy-

totoxic 50% concentration (IC_{50}) was obtained by plotting percent cytotoxicity versus sample concentration (Mostafa *et al*., 2020).

Table 1: Physical and chemical composition (%) of experimental diets used in the experimental stages.

Ingredients	Starter	Grower
	23%	21 %
Yellow corn grains	57.35	62.40
Soybean meal (46.6%)	25.4	23.25
corn Gluten (59.3%)	6.5	3.8
Fish meal (64.7%)	3.5	3.5
Soybean oil	3.5	3.3
Calcium carbonate	1.2	1.2
Di-Calcium Phosphate	1.5	1.5
Common Salt	0.3	0.3
Premix*	0.3	0.3
DL-Methionine,98%	0.2	0.2
Lysine, HCl%	0.15	0.15
Toxenil	0.1	0.1
Total	100	100
Chemical analysis		
ME (kcal /Kg diet)**	3204.83	3202.33
CP,%	22.72	20.513
EE, %	6.138	6.0326
CF, %	2.411	2.398
Calcium%	1.20	1.198
Available phosphorus%**	0.464	0.458
Methionine%**	0.651	0.5487
Lysine%**	1.293	1.0949

*The premix is 3 kg consisted of the following items: Vitamin A (Retinol),120.000.000I.U., Vitamin D3 (Cholecalciferol)20.000.000 I.U., Vitamin K3 (Menadione) 1 g, Vitamin E (aTocopherol acetate) 10 g, Vitamin B1 (Thiamine) 1 g, Vitamin B2 (Riboflavin) 5g, Vitamin B6 (Pyridoxine) 1.5g, Vitamin B12 (Cyanocobalamin) 10 mg, Calcium Pantothenate10g, Niacin (Nicotinic acid) 30g, Folic acid 1g, Biotin 50mg, Manganese60g, Zinc 50 g, Iron 30g, Cupper 4g, Iodine 300mg, Selenium 100 mg, Cobalt 100 mg and Calcium carbonate Add till 3 kg.

Experimental Design

A total of 60 one-day-old commercial Ross broiler chicks were housed in battery cages with regulated light and ventilation in a biosafety level two facility at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP). The chicks were batteries reared, kept under standard hygienic conditions and subjected to a prophylactic vaccination Chicks were vaccinated with the IB- ND live vaccine at 7 days old, the IBD live vaccine at 12 days old, and with IB and Lasota ND live vaccine at 21 days old. The birds were fed on a commercially balanced, antimicrobial-free diet and received unlimited water. The birds in the three experimental groups were fed ad-libitum in two phase feeding programs (starter (3 weeks) and grower (2weeks)). The diet satisfied all the nutritional needs of the chicks by NRC (1994) guidelines as shown in Table 1.

Chicks were randomly divided into three groups, 20 birds each. Group 1 was control group. Group 2 was vaccinated and supplemented with PEE (100% concentration) (1 mL/L drinking water). Group 3 was vaccinated and supplemented with PN (20% concentration) (1 mL/L drinking water). Propolis, either extract or nanoemulsion was supplemented from day one till the end of the experiment at 35 days old in groups 2 and 3, respectively. The weight of birds was recorded from all groups at 21 and 35 days of age to calculate the performance parameters.

Blood samples were collected weekly from each group for five successive weeks for serum separation. Serum samples were used to estimate lysozyme activity, total antioxidant capacity (TAC), and hemagglutination inhibition (HI) against the Newcastle disease virus vaccine (NDVV). At 14, 21, and 28 days old, 3 chicks were humanely euthanized from each group for collection of tissue samples (bursa, spleens thymus, and intestine) to be used for histopathological examination and Molecular detection of spleen gamma interferon (γIFN) and interleukin-2 (IL-2) mRNA gene expression by quantitative real-time PCR (qRT-PCR).

Performance Parameters Data

To assess growth productivity, each group's body weights were recorded at 21 and 35 days of age to determine initial body weight (IBW), final body weight (FBW), and body weight gain (BWG). Feed intake (FI) was calculated by subtracting the leftover feed from the total amount of feed given for each replicate in the treatment group. The feed conversion ratio (FCR) was then calculated for each replicate in the treatment group based on NRC (1994).

Immunological Parameters

Lysozyme assay: Lysozyme activity was measured by agarose gel plate lyses assay according to Schultz (1987). The Lysoplates were prepared by dissolving 1% agarose in 0.06 M PBS (pH 6.3) with 500 mg/L Micrococcus lysodeikticus. The agarose mixture was distributed into plates and 25uL of serum samples and standard lysozyme were added to each well. After 18 h, the diameter of the cleared zones was measured and the lysozyme concentration was estimated from the standard logarithmic curve.

Total antioxidant capacity (tac): It was estimated using the colorimetric method with biodiagnostic kits (CAT. No. TA 2513).

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Table 2: Primers and probes sequences, target genes, and cycling conditions for TaqMan RT-PCR.

Target gene	Primers and probes sequences (5'-3')	Reverse transcription	Primary Denaturation	Amplification (40 cycles)		Reference
				Secondary denaturation	Annealing and extension (Optics on)	
28S RNA	GGCGAAGCCAGAGGAAACT	50°C 30 min.	94°C 15 min.	94°C 15 sec.	60°C 1 min.	(Suzuki *et al.*, 2009)
	GACGACCGATTTGCACGTC					
	(FAM) AGGACCGCTACGGACCTCCAC-CA (TAMRA)					
IFN-γ	GTGAAGAAGGTGAAAGATATCATGGA					
	GCTTTGCGCTGGATTCTCA					
	(FAM) GGCCAAGCTCCCGATGAACGA (TAMRA)					
IL2	TTGGAAAATATCAAGAACAAGAT-TCATC					(Kaiser *et al.*, 2000)
	TCCCAGGTAACACTGCAGAGTTT					
	(FAM) ACTGAGACCCAGGAGTG-CACCCAGC (TAMRA)					

Hemagglutination inhibition (HI) test: The assay was carried out as described by Spackman and Sitaras (2020). Briefly, two-fold diluted serum samples were incubated with 4 HA units of NDV antigen for 30 min at room temperature before 1% prepared chicken RBCs were added. The plates were left at room temperature for 30 min. Results were expressed as positive when hemagglutination was inhibited. Positive and negative control sera were also assessed.

Molecular Detection of Gamma Interferon (IFN-Γ) and Interleukin 2 (IL2) Mrna GENE Expression by Quantitative Real-Time PCR

RNA extraction was applied by using QIA amp RNeasy Mini kit (Qiagen, Germany, GmbH). Extracted RNAs were quantitative before mixing in the RT-PCR reactions. The used oligonucleotide primers and probes are listed in (Table 2) and were supplied from Metabion (Germany). Taqman RT-PCR reaction was performed in Stratagene MX3005p real-time PCR machine. Then analysis of the results to estimate the variation of gene expression was performed. The threshold cycles (CT) of each sample in groups 2 (vaccinated +EEP) and 3 (vaccinated + PN) were compared with that of group 1(vaccinated only) according to the (ΔΔ Ct) method stated by Yuan *et al.* (2006) using the following ratio ($2^{-\Delta\Delta Ct}$).

Pathological Examination

Bursa, spleen, thymus, and intestine were collected from 3 birds from each group at different ages (14, 21, 28 days), fixed in neutral formalin 10% for 48 hours then dehydrated using ethanol alcohol, then embedded in paraffin before obtaining 4-6 μm sections for staining with HandE stain according to Suvarna *et al.* (2018). Semi-quantitative scoring was estimated according to Gibson-Corley *et al.* (2013). Briefly, organs were examined for different parameters (Table 4) and histopathological alterations were divided according to their severity into (0=normal, 1=mild, 2=moderate, 3=severe) and all scores were compared between groups.

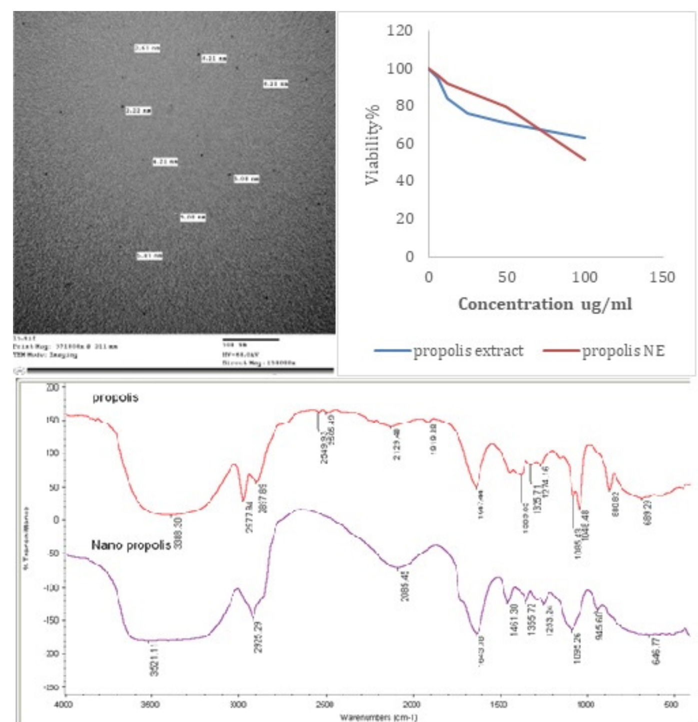

Figure 1 A: HRTEM revealed that droplets size 4.065±1.12 nm, there no aggregation, size homogeneity, and spherical nature. (Central Lab. of Agriculture Collage –Cairo University); **Figure 1B:** Cell viability % of Propolis ethanolic extract and propolis nanoemulsion effect on Vero cells; **Figure 1C:** FTIR of Propolis ethanolic extract and propolis nanoemulsion.

Statistical Analysis

The obtained data were statistically analyzed using a one-way ANOVA test on a computer program using SPSS 14 according to Norusis (2006). Growth parameters data statistically analyzed using a one-way ANOVA test on a computer program using SPSS 21 using Duncan

RESULTS AND DISCUSSION

Preparation and Characterization of PN

Our technique has been modified as involves creating nano-emulsions from propolis using a household blender and a 1500 watt homogenizer, eliminating the need for expensive specialized equipment. PN had a narrow size distribution (4.065±1.12 nm). HRTEM analysis confirmed the absence of aggregation and the presence of size uniformity and spherical morphology with a polydispersity index: of 0.021, while the zeta potential was measured at -10 mV as shown in Figure 1A.

As Functional Principle, the optical bench of the nanoparticle size analyzer NANOTRAC WAVE II is a probe that includes an optical fiber and a Y splitter. Laser light is directed at a volume of sample at the interface of the probe window and the dispersion. The high-reflectivity sapphire window returns a portion of the laser beam to a photodiode detector. The laser light also penetrates the dispersion, and the dispersed light from the particles reflects back at 180 degrees to the same detector. Compared to the reflected laser beam, the sample's dispersed light has a lower optical signal. The high amplitude of the laser beam is added to the low amplitude of the raw scatter signal as a result of the

Table 4: Statistical analysis of pathological alterations between different groups.

Organ	Age /Group								
	14 day			21 day			28 day		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Bursa									
Lymphocytic depletion	2.33 ± 0.58[a]	2.0 ± 00[a]	2.0 ± 00[a]	3.0 ± 00[a]	1.67 ± 0.58[b]	1.33 ± 0.58[b]	2.67 ± 0.58[a]	0.33 ± 0.58[b]	0.33 ± 0.58[b]
edema	2.33 ± 0.58[a]	2.33 ± 0.58[a]	2.67 ± 0.58[a]	3.0 ± 00[a]	1 ± 00[b]	0.33 ± 0.58[c]	1.67 ± 0.58[a]	0.33 ± 0.58[b]	0.33 ± 0.58[b]
Lymphocytic necrosis	1 ± 1[a]	0.33 ± 0.58[a]	0.33 ± 0.58[a]	3.0 ± 00[a]	0.67 ± 0.58[b]	0.33 ± 0.58[b]	3.0 ± 00[a]	0.33 ± 0.58[b]	0.33 ± 0.58[b]
Hyperplasia of lining epithelium	1.33 ± 0.58[a]	1.67 ± 0.58[a]	0.33 ± 0.58[b]	2.67 ± 0.58[a]	0.33 ± 0.58[b]	00 ± 00[b]	2.33 ± 0.58[a]	0.33 ± 0.58[b]	0.33 ± 0.58[b]
Spleen									
Lymphocytic depletion	1.33 ± 0.58[a]	1.67 ± 0.58[a]	1.33 ± 0.58[a]	2.0 ± 00[a]	1.33 ± 0.58[b]	0.33 ± 0.58[b]	3.0 ± 00[a]	2.33 ± 0.58[a]	0.33 ± 0.58[b]
Inflammatory cells infiltration	1.33 ± 0.58[a]	1.33 ± 0.58[a]	1.33 ± 0.58[a]	1.33 ± 0.58[a]	0.67 ± 0.58[a]	0.33 ± 0.58[a]	2.0 ± 00[a]	1.33 ± 0.58[a]	0.33 ± 0.58[b]
Vasculitis	2.67 ± 0.58[a]	2.33 ± 0.58[a]	0.67 ± 0.58[a]	2.66 ± 0.58[a]	0.67 ± 0.58[b]	0.33 ± 0.58[b]	2.33 ± 0.58[a]	0.67 ± 0.58[b]	00 ± 00[b]
Lymphocytic necrosis	0.67 ± 0.58[a]	0.67 ± 0.58[a]	1.33 ± 0.58[a]	2.33 ± 0.58[a]	0.67 ± 0.58[b]	00 ± 00[b]	3.0 ± 00[a]	1.67 ± 0.58[b]	00 ± 00[c]
Thymus									
Hassall's corpuscles	2.33 ± 0.58[a]	1.67 ± 0.58[a]	1.67 ± 0.58[a]	1.67 ± 0.58[a]	1.33 ± 0.58[a]	2.33 ± 0.58[a]	2.33 ± 0.58[a]	2.33 ± 0.58[a]	2.67 ± 0.58[a]
Lymphoid depletion	1.33 ± 0.58[a]	0.67 ± 0.58[a]	1 ± 1[a]	1.33 ± 0.58[a]	1.33 ± 0.58[a]	2.33 ± 0.58[a]	1.67 ± 0.58[a]	2.0 ± 00[a]	2.0 ± 1.0[a]
Hemorrhages	1.67 ± 0.58[a]	1.67 ± 0.58[a]	1.33 ± 0.58[a]	2.33 ± 0.58[a]	1.67 ± 0.58[a]	2.33 ± 0.58[a]	2.67 ± 0.58[a]	2.33 ± 0.58[a]	3.0 ± 00[a]
Intestine									
Inflammatory cells infiltration	2.33 ± 0.58[a]	1.67 ± 0.58[a]	2.0 ± 00[a]	2.67 ± 0.58[a]	1.33 ± 0.58[b]	2.33 ± 0.58[ab]	2.67 ± 0.58[a]	1.33 ± 0.58[b]	2.67 ± 0.58[a]
Necrosis of villi	2.33 ± 0.58[a]	1.33 ± 0.58[a]	1.67 ± 0.58[a]	2.67 ± 0.58[a]	1.33 ± 0.58[b]	2.33 ± 0.58[ab]	2.67 ± 0.58[a]	1.33 ± 0.58[b]	2.67 ± 0.58[a]

Different small letters indicate significantly different between groups within the same row and same age. Data were expressed as mean ± SD at ($P < 0.05$).

reflected laser beam combining with the sample's dispersed light. Other DLS techniques, such as Photon Correlation Spectroscopy (PCS) and NanoTracking (NT), offer a signal to noise ratio up to 106 times lower than that of the Laser Amplified Detection approach.

For TEM, the linear frequency power spectrum obtained from a Fast Fourier Transform (FFT) of the Laser Amplified Detection signal is converted into logarithmic space and deconvoluted to yield the particle size distribution. When used in conjunction with Laser Amplified Detection, this frequency power spectrum calculation offers reliable computation of all particle size distribution types, including narrow, broad, mono-modal, and multi-modal ones. Unlike PCS, which requires a priori information for algorithm fitting, this method does not require such.

Viability Assessment (MTT Assay)

Propolis extract and propolis nanoemulsion exhibited concentration-dependent effects on cell viability. IC_{50} was determined to be 212.7 and 111.5 ug/ml, respectively shown in Figure 1B.

On the confluent surface of Vero cells, results for propolis and nanopropolis 20% had different concentrations (zero (control) 0, 6.25,12.5, 25, 50 and 100 ug/ml with three replicated) after 72hr. of inoculation, the effect on cell viability % was assessed by Mtt assay was 100 ,95.19%, 84.06%, 75.92%,71.18% ,63.25% and 100%, 96.58%, 92.08%, 88.04%, 79.58%,51.88% in 100 ug/ml, respectively.

Hasan *et al.* (2023) reported that the LC_{50} value of propolis was 16.010 ppm, and for 20% NP was 18.689 ppm using Artemia salina Leach larvae with a nanosize is 806.7 nm.

FT-IR Analysis of EEP and PN

Although 20% concentration of propolis extract used in nanoemulsion preparation, the FTIR analysis had small variation between EEP and PN as the following.

- Vibrational frequencies of Propolis extract and propolis nanoemulsion were observed in the range of 3900 to 839 cm^{-1} which more precisely, the functional group spectra that came from the propolis extract's phenolic components were as anticipated.
- In propolis had The O-H stretching of phenolic compounds was assigned to the peaks at 3338.3 cm^{-1}, which were assigned to the elongation of O-H of hydroxyl bonds but 2897.89 and 2977.94 cm^{-1} were assigned to N-H stretching of amino acids, while in nanopropolis had deviate to start from the peaks at 3521.11 cm^{-1} and the N-H was merging in one peak at 2925.29 cm^{-1}.
- The S-H stretching indicated to weak thiol group at 2549.93 and 2505.49 cm^{-1} in propolis which disappear in the nanopropolis.
- The alkene represent at 2129.40, 1919.88 cm^{-1} in propolis which in PN is be 2085.45 cm^{-1}.
- Peak at range 1647.44 and 1643.78 cm^{-1} was presence in EEP and PN that contributed to C=C stretching vibration of vinyl group.
- Propolis extracts and nanopropolis are contain phenolic compounds with functional groups represented by alcohols, carboxylic acids, esters, and ethers, which result in intense patterns between 1389.03 to 1048.48 cm^{-1} that show C–O stretching and C–OH bending.
- FTIR spectra provide the chemical composition, structure, and bonding of nanopropolis which the fingerprint region from 1500-500 cm^{-1} that in propolis ,the vibrations detected at 880.82 and 689.29 cm^{-1} were ascribed to C-H bending and C=C bending represented 1,2,4 trisubstituted and trisubsitituted. Which is the same in nanopropolis is 945.6 and 646.77 cm^{-1} represent to Alkene (C=C bending) and halo compound (C-Br stretching), respectively as shown in Figure 1C.
- Preparation nanopropolis Propolis was crushed using a ball milling process for 24 hours to produce nano-sized particles, with a final size of 58.6 ± 1.1 (Shaheen *et al.*, 2020).

According to Sahlan and Supardi (2013), there was a documented variance in the size of the NP liposome particles, with sizes ranging from 252 to 530 nm. According to measurements, the nano-propolis had a spherical average particle size of 10 nm and a 3 nm standard deviation (Saad *et al.*, 2023). According to Salama *et al.* (2023), the NP particle size varied from 20 to 60 nm, as determined by SEM while the IC_{50} value of NP was 1.7 mg mL^{-1} using human normal (W138) cell lines.

Ay *et al.* (2023) reported that the properties of the size distribution of nanopropolis was a surface potential of −4.21 mV, an average particle size of 59.28 nm, a PDI value of 0.507, vibration peaks at 3269 cm^{-1} that belong to the -OH and -NH groups, peaks of stress vibrations at 2916–2848 cm−1 that belong to the –CH aliphatic group, peaks of –C = O stretching vibrations at 1736 cm^{-1}, peaks of –C = O stretching vibrations at 1603–1462 736 cm^{-1} that belong to the amide or amine group, C–O–C vibration signals at 1162 cm^{-1}, –N-H vibration peaks at 718 cm^{-1}, a sharp peak of the aliphatic groups at 2906 cm^{-1}, weak signals of -C–O–C vibrations at 1270-1163-1064 cm^{-1}, and C = C = C stretching vibrations at 2000cm^{-1} and it is a promising agent for the treatment of breast cancer due to its effectiveness in reducing cell proliferation and inducing apoptosis in breast cancer cells. Woźniak *et al.* (2020) reported that the existence of flavonoid and phenolic chemicals in the propolis extract was suggested by the band at 3369 cm^{-1} in the FTIR spectrum, which corresponds to hydroxyl groups (O-H).

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Oliveira *et al.* (2016) compared propolis from Brazilian propolis (Wax-free) had bands at 3366 and 3273 cm^{-1}, which are associated with hydroxyl groups. Additionally, bands that were somewhat within the same wavelength range were found in other propolis samples. For example, a band at 3487 cm^{-1} was linked to the stretching (v) vibration of hydroxyl groups, while a band at 3433 cm^{-1} was associated to the OH wagging (OH of phenolic compounds). Assigning bands at 2924 cm-1 and 2883 cm-1 to ethanol is possible. The band at 2130 cm-1 was not distinguishable. At 1640 cm-1, a band was discovered. This band may be caused by flavonoids and amino acids: stretching vibration of C=O and C=C, asymmetric bending vibration of N–H], due to C=O stretching vibration of caffeic acid and its derivatives, and due to stretching vibration of C=O of lipids and flavonoids. It may also be caused by aromatic ring deformations. The vibrations of the band at 1457 cm-1 would be the bending (δ) vibration of C–H and the stretching vibration of aromatics, which might be connected to CH3, CH2, flavonoids, and aromatic rings. Non-identifiable bands were also discovered at 1350 and 1289 cm-1. The vibration of the C–O group of polyols, such as hydroxyl flavonoids, would be the cause of the band at 1252 cm-1. A band at 1086 cm-1 might be associated with the C-O-C-O-stretching ester group or secondary alcohols. A band at 878 cm-1 and an unidentified band at 945 cm-1 were present; these bands were most likely caused by ethanol or aromatic ring vibration.

Growth Performance

The obtained results, Table 3, revealed no significant difference (P< 0.05) in the body weight and body weight gain at different experimental periods. Data of the feed intake displayed a significant increase at 21 days old in group 2, and group 3 compared with the control (G1) group. At 35 days old group 3 recorded a significant decrease in feed intake in comparison with group 1 and group 2. Regarding the results of FCR, neither group 2 nor group 3 had an effect at 21 days old. At 35 days old the level of FCR was significantly lowered in group 2 and group 3 than those of group 1.

Adding nano-propolis to broiler drinking water boosted weight gain and feed conversion ratios considerably. The inclusion of propolis to quail meals gave outcomes comparable to drinking water (Denli *et al.*, 2005; Tayeb and Sulaiman, 2014). Consumable components contained in propolis diets, including as resin, wax, honey, and vanillin, may be responsible for the positive impacts on broiler performance (Shalmany and Shivazad, 2006). Propolis has favorable qualities that minimize protein degradation by partially preventing oxidative protein denaturation (Sahin *et al.*, 2003). As a result, the inclusion of nano-propolis in broiler chicken drinking water is expected to improve feed conversion ratios and nutrient digestibility, resulting in superior broiler chicken performance.

Khalati and Al-Salhie (2023), when adding nano-propolis at three doses (50, 100, and 150 µl/liter) to broiler chicken drinking water significantly increased body weight, weight gain, and feed conversion ratio (p ≤0.05). However, there was no effect on cumulative feed consumption.

A dramatic improvement in the growth indices and feed utilization was reported by Eldiasty *et al.* (2024) in poultry supplemented with different concentrations of nanopropolis liposome. Results of body weight disagreed with Refaat *et al.* (2021) who stated the growth-promoting effect of propolis by increasing FBW in poultry. The higher feed intake and poorer feed conversion ratios (FCR) in groups 2 and 3 were in accordance with AL-Kahtani *et al.* (2022). Furthermore, Kishawy *et al.* (2023) noted that nanopropolis liposome could play a crucial role as a growth enhancer to lessen the detrimental effects of base diet on the growth of broiler chickens. Compared to free propolis in earlier studies, the much higher growth rate of broiler chickens under dietary control of nanopropolis liposome can be linked to the combining of propolis into a superior nanoandidate (Aytekin *et al.*, 2020; Kasote *et al.*, 2022).

Immunological Parameters

Lysozyme assay: Using herbals as immunomodulating agents with different vaccination programs in poultry farms

Table 3: Effect of dietary supplementation of EEP and PN on body weight, body weight gain, feed intake and FCR of broilers during experimental period.

Group	Initial weight (g/bird)	Weight 0-21 days	Weight 0-35 days	Body weight Gain 0-21 days	Body weight Gain 0-35 days	Feed in take 0-21 days	Feed in take 0-35 days	FCR 0-21 days	FCR 0-35 days
Group (1) (vaccinated)	43.4± 0.47[b]	913.52 ± 14.87[a]	1692.8 ± 22.10[a]	870.12 ± 14.89[a]	1649.0 ± 7.72[a]	1454.16 ± 7.65[a]	3186.33 ± 8.7[b]	1.59± 0.01[a]	2.16± 0.15[a]
Group (2) (vaccinated + EEP)	39.6± 0.475[a]	919.35 ± 26.11[a]	1759.6 ± 12.68[a]	876.82 ± 27.37[a]	1720.56 ± 8.39[a]	1511.3 ± 4.67[b]	3194.0 ± 3.05[b]	1.76± 0.12[a]	1.69± 0.04[b]
Group (3) (vaccinated + PN)	45.5± 0.85[c]	871.56 ±13.17[a]	1647.2 ± 13.32[a]	829.25 ± 24.92[a]	1602.1 ± 5.28[a]	1498.63 ± 0.92[b]	3066.33 ± 1.77[a]	1.59± 0.26[a]	1.59± 0.06[b]

a,b, Means (Mean± standard Error) within the same column with different superscripts are significantly differ (P ≤ 0.05).

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are essential to minimize the cost of vaccination, improve the health status of birds, and reduce some disease conditions (Eladl *et al*., 2020).

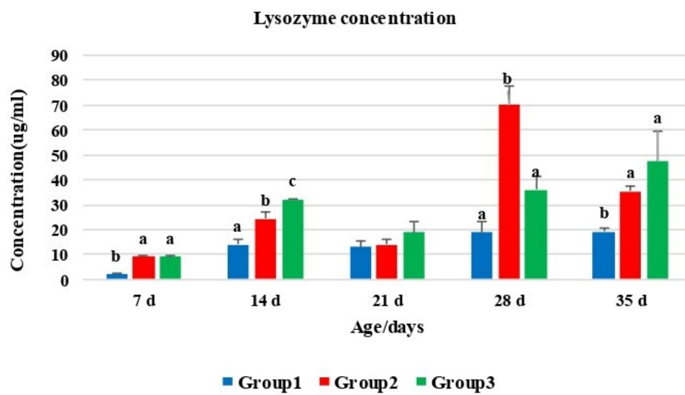

Figure 2: Effect of propolis ethanolic extract and propolis nanoemulsion on Lysozyme concentration at different ages. Different small letters indicate significant differences between groups within the same period. Data were expressed as mean ± SE at (P < 0.05).

The obtained data Figure 2, revealed a significant increase in lysozyme concentration in group 2 (vaccinated + EEP) at 7,14,28 and 35 days' old compared to the vaccinated group (group 1). Those of group 3(vaccinated + PN) showed a significant increase in lysozyme at 7,14 and 35 days old in comparison with the vaccinated group (group 1). Comparing group 2 (vaccinated + EEP) and group 3(vaccinated + PN) declared that group 3(vaccinated + PN) had a significant and earlier effect in the elevation of lysozyme concentration that recorded 32 ($p < 0.05$) at 14 days old. However, those of group 2 (vaccinated + EEP) recorded a significant elevation that reached 70 ($p<0.05$) at 28 days old.

The finding reflects the boosting immunomodulatory effect of propolis extract on lysozyme concentration by improving the total leukocytic count and phagocytic activity (Mona *et al*., 2021). Such improvement is referred to the propolis content of many chemical compounds with immunostimulant effects such as flavonoids, polyphenols, aromatic acids, alkaloids, and terpenoids (Abdel-Maksoud *et al*., 2023). Ahangari *et al*. (2018) stated that the biological functions of propolis were achieved through its components of aromatic, phenolic, and flavonoid compounds. Nanoparticles resulted in macrophage activation after being bonded with the receptors expressed on the surface of macrophage (Liu *et al*., 2022). Such activation leads to production of large amount of lysozyme which had a vital role in innate immunity including, immuneomodulatory, antiviral, antibacterial and anti-inflammatory. Therefore, it offered protection before activation of specific immunity. Hence, it covered the immunological gap (El-Sissi *et al*., 2020).

Total antioxidant capacity: Figure 3, exhibits the finding of (TAC). The values displayed the successful effect of vaccine and propolis, either as ethanolic extract (group 2) or as nanoemulsion (group 3) in improving TAC at 14 and 35 days old in comparison with the vaccinated group only (group 1). At 14 days old group 2(vaccinated + EEP) recorded a significant elevation in TAC compared with group 3(vaccinated + PN).

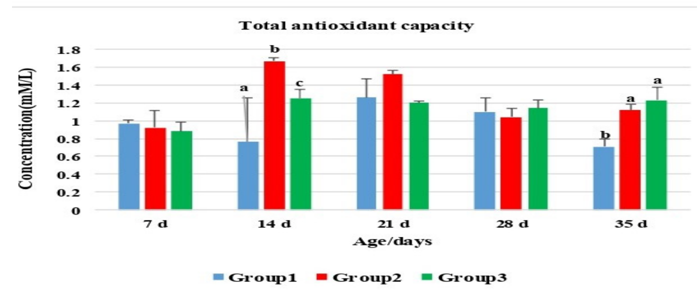

Figure 3: Effect of propolis ethanolic extract and propolis nanoemulsion on total antioxidant capacity concentration at different ages. Different small letters indicate significant differences between groups within the same period. Data were expressed as mean ± SE at (P < 0.05).

Free radicals have the ability to cause many disorders and weaknesses in different body systems (Nayak *et al*., 2023). The high phenolic content of propolis has the ability to scavenge free radicals resulting in elevation of TAC and decreasing malondialdehyde (MDA) (Abbas *et al*., 2020). Hotta *et al*. (2020) stated the activation of the intracellular antioxidant pathway of Nrf2-ARE by the action of the ethanolic extract of Brazilian red propolis. The Nrf2-ARE pathway plays a defensive role in diminishing oxidative stress. This explains the role of nuclear factor E2-related factor (Nrf2) in emphasizing the expression of huge numbers of cytoprotective and detoxificant gens (Buendia *et al*., 2016). The small size of nanopropolis makes it preferable to be used as an antioxidant and antimicrobial agent (Bezerra *et al*., 2023). The hepatoprotective effect of Bulgarian nanopropolis was manifested by restoring levels of glutathione, normalization the activity of serum transaminase and a significant protection against liver antioxidant enzymes SOD and CAT (Tzankova *et al*., 2019).

Hemagglutination inhibition (HI) test: The results of HI against NDVV were expressed as log. 2 antibody titer, Figure 4. Supplementation of vaccinated chicken with propolis as ethanolic extract (group 2) or as a nanoemulsion (group 3) did not influence the antibody titer against NDVV throughout the experiment. The expressed data is compatible with Eyng *et al*. (2015) and differ from those of Mona *et al*. (2021) who observed the positive effect of propolis in improving antibody titer against the Newcastle disease vaccine in broiler chickens. This discrepancy may be due to differences in propolis composition, dosage or the experimental condition of the studies.

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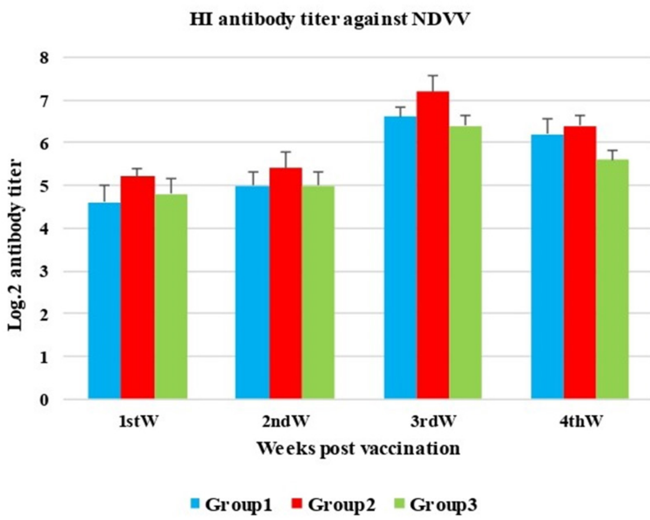

Figure 4: Effect of propolis ethanolic extract and propolis nanoemulsion on HI antibody titer against NDVV at different ages. Data were expressed as mean ± SE at ($P < 0.05$).

Relative Expression of IFN-r and IL2 Mrna Genes by Quantitative Real-Time PCR

The fold changes for (IFN-γ) and (IL2) m RNA gene expression were presented in (Figures 5 and 6). The expression of both genes was significantly increased ($p< 0.05$) in group 2 and group 3 at 14, 21, and 28 days old in comparison with group 1. Moreover, the gene expressions were upregulated in group 3 compared with those of group 2 at all intervals. The obtained data are like Abdel-Maksoud *et al.* (2023) who recorded a significant increase in gene expression of INF-γ and IL2 in poultry supplemented with PE in drinking water. Additionally, PE activates the avian TLR3 which initiates transcription of interferon's genes (Vickers, 2017).

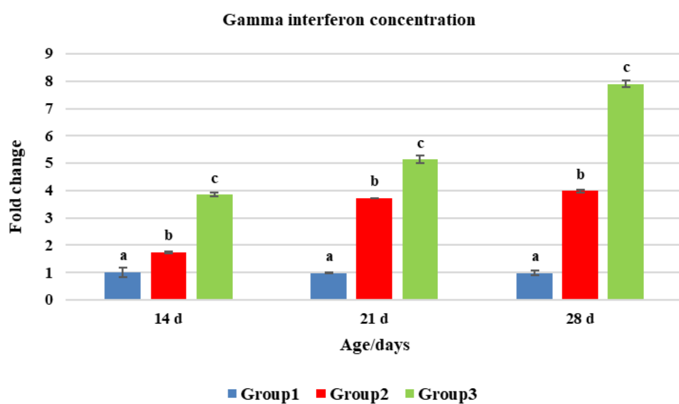

Figure 5: Effect of propolis ethanoic extract and propolis nanoemulsion on mRNA gene expression of γ interferon. Different small letters indicate significantly different between groups within the same period .Data were expressed as mean ± SE at ($P < 0.05$).

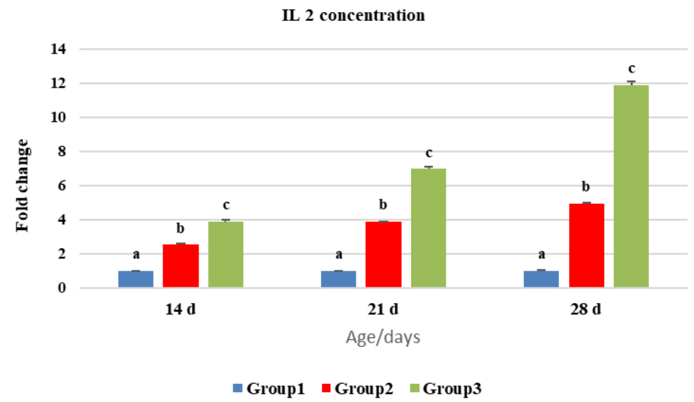

Figure 6: Effect of propolis extract and propolis nanoemulsion on mRNA gene expression of IL-2. Different small letters indicate significantly different between groups within the same period. Data were expressed as mean ± SE at ($P < 0.05$).

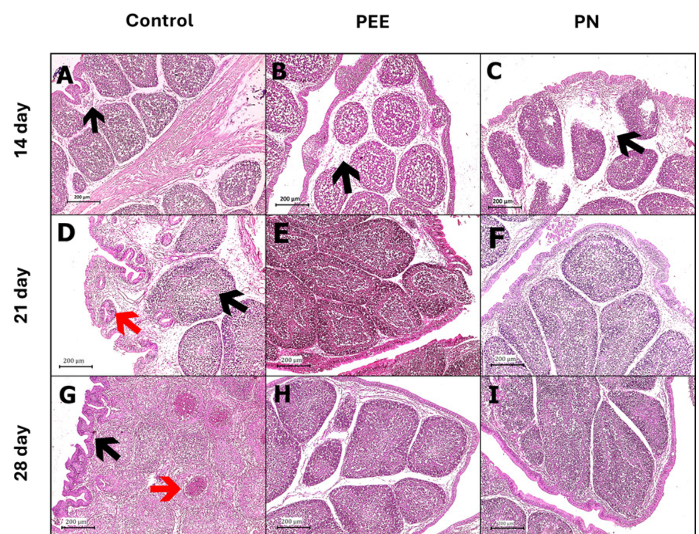

Figure 7: photomicrograph of bursa (HandE); 14 day; mild lymphoid depletion and interlobular edema (arrow) (A) – mild lymphoid depletion, hyperplasia of cortical epithelium and perifollicular edema (arrow) (B) – marked bursitis with lymphoid depletion and severe edema (arrow) (C) – 21 day; noticeable medullary depletion (black arrow) and hyperplasia of cortical epithelium (red arrow) (D) – slight lymphoid depletion of the medulla (E and F) – 28 day; marked lymphoid follicle with follicular cyst formation (red arrow)and hyperplasia of cortex(black arrow) (G) – normal bursal architecture with normal diffuse lymphoid follicles and outer cortices (H and I).

Pathological Findings

Bursa of group 1 showed mild lymphocytic depletion with perifollicular edema at 14d (Figure 7A). On 21d, the severity of bursitis and lymphoid depletion was noticed with hyperplasia of the cortical epithelium (Figure 7D). One week later, the bursa revealed mild follicular necrosis and follicular cysts formation (Figure 7G). The bursa of group 2 and group 3 at the age of 14 d showed comparable pathological alterations as group 1 (Figure 7B and 7C), while

improvement with normal bursal architecture was noticed in examined bursa on 21d and 28d age (Figure 7E,7F,7H and 7I). The spleen of groups 1and2 revealed mild lymphoid depletion with severe vasculitis (Figure 8A and 8B) while group 3, showed just focal lymphoid depletion (Figure 8C) at 14d age. On 21d, the spleen of group 1 showed the same alterations of depletion and multiple vasculitis (Figure 8D); while groups 2 and 3 showed noticeable normal architecture and lymphocytes (Figure 8E and 8F). On 28d, the spleen of group 1 showed necrosis of the lymphocytes and thickening of the splenic capsule (Figure 8G), while group 2 showed multiple lymphoid depletion (Figure 8H). On the other hand, group 3 revealed normal splenic architecture (Figure 8I).

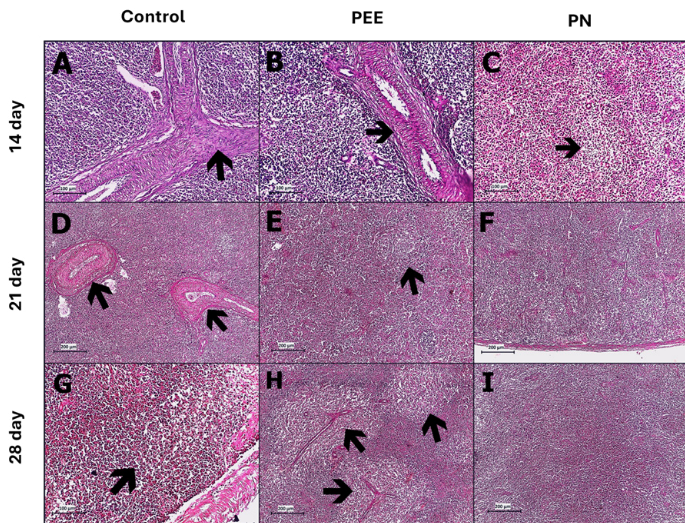

Figure 8: photomicrograph of spleen (HandE); 14 day; splenitis with mild lymphoid depletion and noticeable vasculitis (arrow) (A and B) – focal lymphocytic depletion and necrosis of lymphocytes (arrow) (C) – 21 day; multiple vasculitis (black arrow) and mild lymphoid depletion (D) – normal splenic architecture with negligible focal lymphoid depletion (E and F) – 28 day; marked lymphocytic necrosis (black arrow) with thickened capsule (G) – multiple lymphocytic depletion of lymphoid nodules (H) – normal splenic architecture and lymphoid aggregation (I).

Thymus from chickens at 14d age of all groups showed lymphoid depletion in the thymic medulla with the increased number of Hassall's corpuscles (Figure 9A, 9B and 9C). At 21d age, the thymus of group 1 showed lymphoid depletion in the cortex and medulla with hemorrhages and an increased number of Hassall's corpuscles (Figure 9D) while the thymus of group 2 showed mild focal lymphoid depletion in the cortex and medulla (Figure 9E). In case of group 3, thymus revealed noticeable depletion in the medulla with congested blood vessels and increased number of Hassall's corpuscles (Figure 9F). At 28d age, thymus of all groups revealed pathological alterations with lymphoid depletion in cortex and medulla (Figure 9G, 9H and 9I). The intestine of group 1 over moderate enteritis at all examined ages with massive destruction of the intestinal villi and marked lymphocytic cell infiltration (Figure 10A, 10D and 10G). The intestine of group 2 showed mild enteritis with frequently intact villi and few lymphocytic cell infiltrations (Figure 10B, 10E and 10H). Whereas, the intestine of group 3 showed marked enteritis with destructive villi, lymphocytic cell infiltration, and hyperplasia of the intestinal gland (Figure 10C, 10F and 10I).

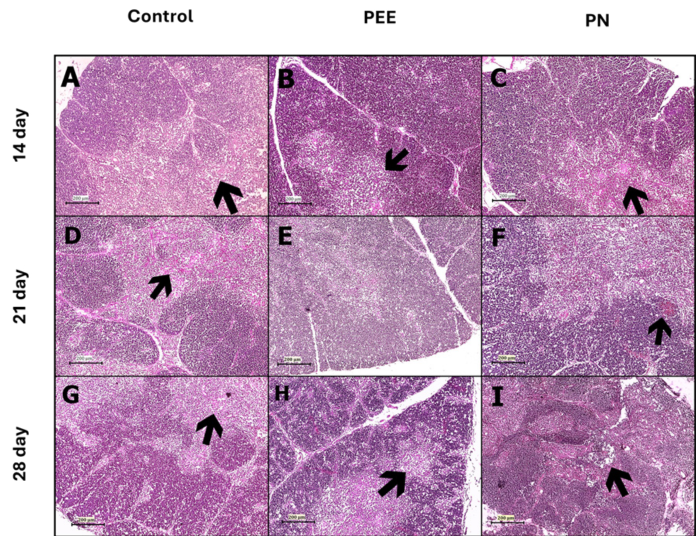

Figure 9: photomicrograph of thymus (HandE); 14 day; mild lymphoid depletion of thymic medulla with increased number of Hassall's corpuscles (arrow) (A,B,C)– 21 day; lymphoid depletion in cortex and medulla with medullary hemorrhages (arrow)(D) – focal lymphoid depletion in cortex and medulla (E) – marked lymphoid depletion, congestion of blood vessels (arrow) and increased number of Hassall's corpuscles(F) -28 day; marked lymphoid depletion in cortex and medulla (G,H,I).

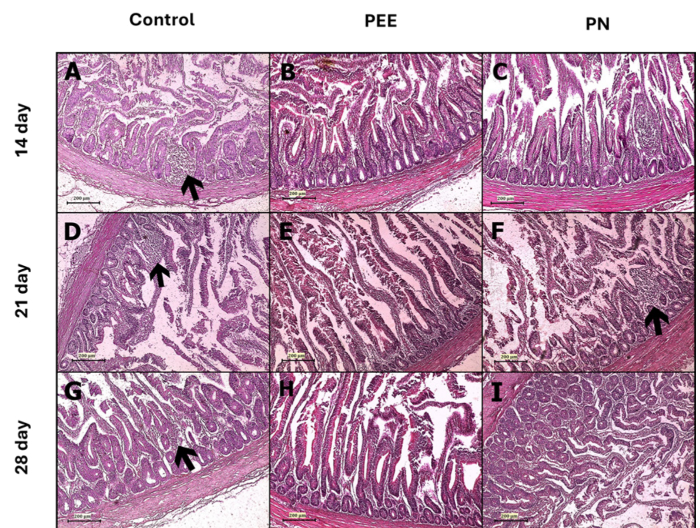

Figure 10: photomicrograph of intestine (HandE); moderate enteritis with destruction of the villi and focal lymphocytic cells infiltration (arrow) (A, D, G), mild enteritis with frequently intact villi and few lymphocytic cells infiltration (B, E, H), marked enteritis with destructed villi and lymphocytic cells infiltration (arrow) (C,F,I).

The statistical analysis of pathological alterations revealed significant pathological differences between examined groups which revealed noticeable improvement of immune organs (bursa and spleen) and intestinal morphology of supplemented groups either with PEE or PN (Table 4). These pathological findings were somewhat confirmed by Abdel-Maksoud *et al.* (2023) who found normal lymphocytes in the medullae of the bursa, spleen, and thymus, of propolis-supplemented chickens. Moreover, our finding in pathological intestinal improvement was agreed by Mona *et al.* (2021) who previously stated a marked increase in intestinal villi length and width with intact epithelium and hyperplasia of intestinal glands of propolis-supplemented broiler chickens compared to control. In addition, (Eldiasty *et al.*, 2024) described the beneficial effect of nanopropolis on chickens exposed to cyclic heat stress, which improves intestinal architecture by enhancing blood metabolites and immunity, reducing inflammation and oxidative stress.

CONCLUSIONS AND RECOMMENDATIONS

Propolis and nano propolis exhibited an obvious immunomodulatory effect on the vaccine, increasing interleukins IL-2 and IFN-γ by at least 2-fold compared to the vaccine alone. Additionally, there was an increase in feed intake during the first three weeks of age. Therefore, propolis seems to be a harmless and effective feed supplement in the chicken diet. Also, nanopropolis could be used as a safe growth promoter and immunological enhancer in poultry farms.

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NOVELTY STATEMENT

Related to the bioactive compounds present in propolis, such as flavonoids, phenolic acids, and terpenoid, Exhibit antimicrobial activity, reducing the load of pathogens in the gut and promoting a healthy gut microbiome. Inhibit inflammation and oxidative stress, which can improve nutrient absorption and overall health. Stimulate the immune system especially to cellular immunity which enhancing the body's natural defenses to reduce side effect of vaccine

AUTHOR'S CONTRIBUTIONS

All authors contributed equally.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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