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Article

Supplementation of broiler diets with propolis and oregano oil and its effect on production parameters, leukocytes, metabolites and breast meat antioxidant stability

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Abstract:

Propolis and oregano oil are natural substances used in various food industry applications. An evaluation was done of the effects of oregano oil (A) and propolis (P) on production parameters, leukocytes, blood chemistry and meat antioxidant stability in broilers. Animals (n= 480) were randomly allocated to four treatments with four replicates of 30 animals each. Four additive levels (mg/k feed) were tested: C (control)= 0; P= 100 mg propolis; A= 100 mg oregano oil; and AP= 50 mg P + 50 mg A. At 42 d breast meat lipid oxidative stability was estimated based on malondialdehyde (MDA) content. The oregano oil contained 43.47% thymol and 29.16 % carvacrol, while the propolis contained 5.6 mg flavonoids, 840 µg phenols and 138 µg Trolox® equivalents (antioxidant stability) per gram. Feed intake, weight gain, feed conversion and mortality were unaffected by the dietary additives. At 3 wk, blood

eosinophil levels increased in treatment AP ($P \leq 0.05$), and at 6 wk triglycerides had increased in treatment A ($P \leq 0.05$). Meat lipid oxidative stability decreased in the AP treatment ($P \leq 0.05$). Neither oregano oil nor propolis improved production parameters, although they can stimulate immune response. When added to low-fat broiler diets they can increase blood triglycerides and in combination they compromise breast meat lipid oxidative stability.

Key words: Essential oils, Natural additives, Broilers, Oxidative stability.

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Introduction

Restrictions on the use of synthetic substances in animal production systems, including of poultry species, has driven increasing interest in natural substances as growth stimulants[1,2]. These can function by increasing production parameters, supporting immune response, improving health condition and decreasing oxidation of fats in chicken meat[3,4].

Oregano oil is used as a preservative in the food industry because it prevents microorganism growth[5-7]. Its main compounds are thymol and carvacrol, which can represent up to 80 % of its content and are responsible for its biological activity[8,9].

Propolis is produced by bees from resins collected from trees, shrubs and plants. It has myriad functions in the beehive, including as an antiseptic to prevent the growth of microorganisms in the hive. Over 300 substances have been identified in propolis such as aromatic acids, diterpenes, phenols and flavonoids[10-13], all of which are bioactive compounds with anticancer, anti-inflammatory, bactericidal, viricidal, immunostimulatory and antioxidant capacity both *in vivo* and *in vitro*[14-16].

Due to their price, ease of use and benefits oregano oil and propolis are currently in use as alternative compounds in the poultry industry, however results vary in response to their sources[17-19]. Combining natural bioactive compounds can potentiate their biological effects, increasing organism response. The present study objective was to evaluate if oregano oil alone or in combination with propolis affects productive variables, blood leukocytes, lymphocytes and chemical element concentrations, and oxidative stability of broiler breast meat.

Material and methods

All animal protocols in the present study comply with animal welfare guidelines and were approved by the Animal Care and Use Committee of the University of Nayarit (Tepic, Mexico).

Oregano essential oil composition

Compounds in the oregano oil were identified with a gas chromatographer (GC; Hewlett Packard P-6890, California, USA) attached to a mass spectrophotometer (MS; Hewlett Packard 7953, California, USA), using a capillary column (30 m long, 0.25 mm internal diameter, 0.25 µm film thickness; Hewlett Packard 5ms®, California, USA). Injection port temperature was 240 °C. Initial temperature was 50 °C for 5 min, and was increased 10 °C per minute until reaching 260 °C. The carrier gas was helium. The MS was run in scan mode (m/z range: 30-550) with electronic ionization (70 eV) and a 1.0 ml/min flow rate.

Propolis flavonoids and phenols contents and antioxidant capacity

Flavonoids content was quantified following the aluminum chloride method, total phenols with the Folin-Ciocalteu method and antioxidant capacity with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method[20].

Experiment location

The evaluation was done at the Veterinary Medicine and Zootechny Academic Unit poultry farm of the Universidad de Nayarit (Compostela Municipality, Nayarit, Mexico).

Animals and experimental diets

Experimental animals were 480, one-d-old Ross line broilers randomly distributed in four treatments (C= control, P= 100 mg propolis/kg feed, A = 100 mg oregano oil/kg feed, PA=

50 mg propolis plus 50 mg oregano oil/kg feed) with four replicates of 30 chickens each per treatment. Starter and finishing diets were based on corn-soybean paste meal (Table 1) provided ad libitum for 42 d. The oregano oil and/or propolis were added during feed processing.

Table 1: Experimental diet composition (%)

Ingredients	Starter	Growth / Finishing
Corn	65.41	72.16
Soy paste	29.22	22.11
Raw soy oil	1.00	1.86
Calcium bicarbonate (38%)	1.64	1.52
Dicalcium phosphate (18/21)	1.49	1.30
Salt	0.30	0.30
Mineral and vitamin premix[1]	0.30	0.30
DL-Methionine	0.30	0.18
HCL-Lysine	0.29	0.19
Xanthophylls[2]	0.00	0.03
Coccidiostat	0.05	0.05
	100.00	100.00
Nutrient composition:		
Mcal/kg	3.00	3.10
Crude protein	20.06	17.00
Calcium	1.00	0.90
Lysine	1.30	1.00
Methionine + Cysteine	0.95	0.75
Methionine	0.50	0.40
Available phosphorous	0.45	0.45
Histidine	0.51	0.43
Tryptophan	0.27	0.23
Threonine	0.84	0.73
Arginine	1.31	1.08
Linoleic acid	1.90	2.46

[1] Vitamin premix (/kg feed): vitamin A, 10,000 UI; vitamin D_3, 2,500 UI; vitamin K_3, 2 mg; thiamin, 2 mg; riboflavin, 7 mg; pantothenic acid, 10 mg; pyridoxine, 4 mg; folic acid, 1 mg; vitamin B_{12}, 0.015 mg; and biotin, 0.010 mg (Vipresa®, Tepatitlán de Morelos, Mexico). Mineral premix (mg/kg feed): Se, 0.20; I, 0.30; Cu, 7; Fe, 65; Zn, 75; Mn, 65; and Co, 0.4 (Vipresa®, Tepatitlán de Morelos, Mexico).

[2] 90 ppm *Tagetes erecta* (Florafil-93 Powder, Industrias Vepinsa S.A. de C.V., Los Mochis, Sinaloa, Mexico).

Productive parameters and blood samples

Productive parameters were measured every seven days, and mortality as it occurred. At d 21 and 42, blood samples were taken from the brachial vein of two birds per replicate and 1.8 mg ethylenediaminetetraacetic acid (EDTA) per ml added. These samples were dyed with Wright dye for blood metabolite measurement (Easy-Vet, Desego).

Meat samples

Meat samples were taken from two birds per replicate. After a 6 h fast, the animals were killed by severing the jugular vein and carotid artery following an established protocol (Norma Oficial Mexicana NOM-033-SAG/ZOO-2014)[21]. The carcass was drained of blood for 2 min, placed in water (60 °C) for 120 sec to allow for manual removal of the feathers, and cooled in ice water (0 °C) for one hour. The breast meat was removed from the carcass, the skin and any visible fat removed, and the meat stored in a vacuum and frozen (-20 °C) for approximately one month.

Meat oxidative stability

Oxidative stability was measured using a 30 g sample of meat and adding 30 ml distilled water with 0.2 mL 7% BHT (2,6-di-tert-butyl-4-methyl-phenol, Sigma-Aldrich, Toluca, Mexico) diluted in 96% methyl alcohol (CH_3CH_2OH). This mixture was liquified for 30 sec in a blender (Oster, M4655-813 / 465-42), filtered through a 0.84 mm plastic mesh and left to stand for 30 min at 25 °C in darkness. A 1 mL subsample was taken from the top layer and 2 ml 0.02 M thiobarbituric acid (Sigma-Aldrich, Toluca, Mexico) combined with 15% trichloroacetic acid (TCA) in distilled water added to it. The solution was stirred for 10 sec, placed in water at 80 °C for 10 min and finally at 0 °C for 10 min. Absorbance was measured at 532 nm (Biotek, Epoch, USA), and the resulting values multiplied by 7.8 to express mg malondialdehyde (MDA) per kilo of meat[22].

Statistical analysis

The results were analyzed with a completely random design using the Generalized Linear Method (GLM) of the SAS statistics program. The means were compared with a Tukey test at a $P \leq 0.05$ significance level. Mortality results were transformed with an arc sine function and expressed as a percentage. The statistical model was:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where:

$\mathbf{Y_{ij}}$ is feed intake, weight gain, feed conversion, mortality, leukocytes, chemical variables and MDA per kilogram of meat;
$\boldsymbol{\mu}$ is the general mean;
$\boldsymbol{T_i}$ is the effect of the oregano oil, propolis and oregano oil plus propolis;
$\boldsymbol{E_{ij}}$ is the random error.

Results and discussion

Oregano oil

The most abundant compounds in the oregano oil were thymol (43.47 %), carvacrol (29.16 %), eucalyptol (6.96 %), caryophyllene (5.38 %) and tetramethyl (2.96 %). It is the thymol and carvacrol contents of oregano oil that slow bacterial growth. Their concentrations can vary due to place of origin, harvest time and plant maturity, but they remain the most abundant compounds in all phenological stages of the oregano plant. These two compounds can account for as much as 80% of oregano oil[23,24]. In the present study they accounted for 72 %, more than twice that reported elsewhere[25,26].

Propolis

Flavonoid content in the evaluated propolis was 5.6 mg quercetin equivalents/g propolis, phenols content was 840 µg caffeic acid equivalents/g propolis, and antioxidant capacity was 138 µg Trolox equivalents/g propolis. Biologically active compound contents can vary widely in propolis depending on source region; for example, flavonoid contents ranging from 8 to 188 mg/g propolis and phenols contents ranging from 42.9 to 329.0 mg/g propolis have been reported from China, India, Macedonia and Iran[10,24,25]. Reported levels in propolis from Mexico are 379.2 mg flavonoids, 500.9 mg phenols and 54.4 mg Trolox equivalents per gram of propolis[20]. Compared to the above values, the propolis analyzed here contained limited amounts of these active compounds.

Productive parameters

Inclusion of the oregano oil and/or propolis in the feed had no effect on feed intake, weight gain, feed conversion and mortality (Table 2). Apparently, the animals exhibited no quantifiable response because the inclusion levels were not high enough. Previous reports of productive performance in broilers in response to addition of oregano oil or propolis in poultry diets are variable. In two studies inclusion levels of 25, 50, 300 and 600 ppm propolis per kilogram of feed had no effect on broilers[27,28]. In another study, only addition of 15,000 and 20,000 ppm oregano oil to boiler diets increased productive yields[29]. In the present study the doses of propolis and/or oregano oil did not provide enough active compounds to stimulate digestive enzyme secretion, and the birds consequently showed no changes in productive parameters. Only high doses or larger amounts of active compounds can stimulate digestive enzyme secretion, allowing the birds to better exploit ingested nutrients and thus increase yield[30]. A serious challenge when comparing studies of production performance in response to natural compounds is that most do not report the amount of active compounds contained in evaluated propolis or essential oils, precluding any comparisons.

Table 2: Productive parameters (kg) of broilers at three and six weeks of age

Treatments	Feed intake	Weight gain	Feed conversion	Mortality (%)
	Three weeks			
C	0.68 ± 0.03	0.43 ± 0.02	1.58	4.20
A	0.66 ± 0.01	0.42 ± 0.02	1.59	7.50
P	0.67 ± 0.01	0.43 ± 0.01	1.58	10.00
AP	0.69 ± 0.01	0.43 ± 0.01	1.59	9.20
SME	0.004	0.003	0.011	0.453
	Six weeks			
C	3.99 ± 0.16	2.01 ± 0.02	1.99	3.30
A	3.74 ± 0.14	1.89 ± 0.12	1.98	1.70
P	3.65 ± 0.24	1.80 ± 0.14	2.03	1.70
AP	3.79 ± 0.09	1.89 ± 0.09	2.01	5.00
SME	0.049	0.030	0.017	0.181

C= control; A= oregano oil (100 mg kg^{-1} feed); P= propolis (100 mg kg^{-1} feed); AP= oregano oil (50 mg kg^{-1} feed) + propolis (50 mg kg^{-1} feed).
SME= Standard mean error.

Leukocytes

At three weeks, lymphocyte levels were lowest in the P treatment ($P \leq 0.05$), and eosinophils were highest in the AP treatment ($P \leq 0.05$). The C and A treatments were similar (Table 3). Lymphocytes support immune response when challenged by invading microorganisms. The present results coincide with previous studies in which lymphocyte levels in poultry decreased in response to addition of propolis to the diet because the active compounds in propolis inhibit development of T lymphocytes[31,32]. In another study levels as low as 5 µg propolis per milliliter caused negative effects *in vitro*, due to the flavonoids content[33].

Eosinophil levels were two-fold higher in the AP treatment than in the P treatment. Eosinophils are cells linked to the development of T lymphocytes, the populations of which decrease as bird age increases[34]. However, when development of the digestive system improves, eosinophils increase; broilers offered feed with added oregano oil or propolis have exhibited improved intestinal flora and stimulation of cytosines which induced eosinophil proliferation[35].

Table 3: Blood leukocyte concentrations in broilers at three and six weeks of age

Treatment	Lymphocytes	Heterophils	Eosinophils	Basophils	Monocytes
	Three weeks				
C	68.8 ± 3.1 a	29.8 ± 2.4	0.0 ± 0.0 c	0.5 ± 0.5	0.3 ± 0.5
A	61.8 ± 2.9 ab	36.9 ± 13.3	0.0 ± 0.0 c	0.0 ± 0.0	0.0 ± 0.0
P	55.2 ± 3.3 b	43.2 ± 3.1	1.5 ± 1.4 b	0.3 ± 0.5	0.0 ± 0.0
AP	57.9 ± 8.9 ab	38.5 ± 10.1	3.6 ± 1.3 a	0.4 ± 0.5	0.0 ± 0.0
SME	1.65	1.67	0.313	0.083	0.043
	Six weeks				
C	42.0 ± 10.2	19.6 ± 4.4	27.3 ± 8.0	2.8 ± 2.6	8.4 ± 7.7
A	38.4 ± 12.4	18.1 ± 7.8	31.0 ± 11.3	3.6 ± 2.9	12.0 ± 6.1
P	45.3 ± 10.1	14.8 ± 3.0	3.5 ± 2.3	3.5 ± 2.3	12.9 ± 3.9
AP	51.6 ± 9.4	17.5 ± 10.2	43.9 ± 63.0	2.4 ± 2.6	6.4 ± 4.6
SME	1.869	1.154	5.558	0.730	1.504

C= control; A= oregano oil (100 mg kg^{-1} feed); P= propolis (100 mg kg^{-1} feed); AP= oregano oil (50 mg kg^{-1} feed) + propolis (50 mg kg^{-1} feed).

SME= Standard mean error.

[abc] Different letter suffixes in the same column and age group indicate significant difference ($P<0.05$).

Blood metabolites

In six-week-old birds, triglycerides levels were highest in treatment A ($P \leq 0.05$) and lowest in AP, while C and P had intermediate values and did not differ between themselves (Table 4). Blood metabolite levels are indicative of general animal health. Addition of propolis (300 ppm) in broiler diets has been reported to lower cholesterol and triglyceride levels[36], although this response is inconsistent[37]. For example, broilers fed diets containing 6% fat levels and added propolis exhibited low cholesterol and triglyceride levels[38], whereas in trials with lower energy levels no hypocholesterolemic or hypolipidemic effect was observed with addition of propolis or oregano oil to the diet.

Table 4: Blood metabolites levels in broilers at three and six weeks of age

Metabolite	C	A	P	AP	SME
			Three weeks		
Glucose	137.8 ± 41.3	111.8 ± 58.4	106.3 ± 38.6	105.1 ± 49.5	8.00
Urea	6.2 ± 2.7	5.9 ± 1.5	4.8 ± 2.1	3.9 ± 0.9	0.00
Uric acid	5.6 ± 1.3	4.1 ± 1.4	5.6 ± 2.4	5.3 ± 2.2	0.00
Creatine	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.00
Cholesterol	234.6 ± 22.5	229.9 ± 41.6	237.3 ± 36.0	263.3 ± 35.6	6.00
Triglycerides	96.9 ±23.4	84.5 ± 21.0	84.3 ± 12.9	78.4 ± 9.4	3.00
			Six weeks		
Glucose	284.8 ± 55.6	265.9 ± 58.8	313.8 ± 84.0	279.6 ± 27.4	10.478
Urea	3.1 ± 1.8	3.1 ± 3.1	2.9 ± 1.9	2.9 ± 1.4	0.356
Uric acid	7.4 ± 3.2	11.0 ± 7.3	7.7 ± 5.4	7.6 ± 2.2	1.000
Creatine	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.200
Cholesterol	170.4 ± 26.3	189.0 ± 51.4	186.5 ± 37.5	167.8 ± 30.6	0.516
Triglycerides	55.1 ± 10.0 ab	68.8 ± 22.9 a	50.3 ± 6.4 ab	45.0 ± 11.5 b	2.836

C= control; A= oregano oil (100 mg kg^{-1} feed); P= propolis (100 mg kg^{-1} feed); AP= oregano oil (50 mg kg^{-1} feed) + propolis (50 mg kg^{-1} feed).

SME= Standard mean error.

[abc] Different letter suffixes in the same row indicate significant difference ($P<0.05$).

Chicken breast oxidative stability

Oxidation in the breast meat was highest in the AP treatment ($P \leq 0.05$), with no difference between the A, P and C treatments (Table 5). Propolis and oregano oil are known to have antioxidant capacity *in vitro* and *in vivo*[16,39], but their effects may vary when mixed with other ingredients in a diet. For example, oregano oil combined with acidified soybean oil has no antioxidant effect[39], whereas addition of 200 ppm propolis is reported to decrease MDA content in chicken meat[40]. The latter may be due to accumulation of the bioactive compounds in propolis in cell membranes, which could protect them from oxidation[33,36]. Apparently, combining oregano oil with propolis accelerates the oxidative process in meat, but when administered independently they have no effect on MDA levels.

Table 5: Oxidative stability in chicken breast meat (malondialdehyde per kilogram of meat)

Treatment	Mean ± standard deviation
C	0.849 ± 0.34 b
A	1.116 ± 0.41 b
P	0.670 ± 0.39 b
AP	1.864 ± 0.58 a
SME	0.262

C= control; A= oregano oil (100 mg kg^{-1} feed); P= propolis (100 mg kg^{-1} feed); AP= oregano oil (50 mg kg^{-1} feed) + propolis (50 mg kg^{-1} feed).

SME= Standard mean error.

[abc] Different letter suffixes in the same column indicate significant difference ($P<0.05$).

Conclusions and implications

The evaluated oregano oil contained 43.47 % thymol and 29.16 % carvacrol, and the propolis contained 840 µg phenols, 5.6 mg flavonoids and 138 µg Trolox equivalents. When added to broiler diets either alone or in combination they produced no increase in productive performance. At three weeks age, overall white blood cell counts decreased in the different treatments, although eosinophil counts increased. At six weeks, blood triglycerides increased in the oregano oil treatment. Combining oregano oil with propolis increased oxidation in breast meat. The present results do not indicate any clear benefit from including oregano oil and/or propolis in broiler diets at the evaluated concentrations. Further research is needed to

identify at what concentrations these natural substances exercise a positive effect on productive performance, and if combining them could improve broiler productivity and health.

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