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Anti-inflammatory Effects of *Chrysactinia mexicana* Gray Extract in Growing Chicks (*Gallus gallusdomesticus*) Challenged with LPS and PHA

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The effect of *Chrysactinia mexicana* extract on broilers challenged with LPS and PHA was evaluated. A two 2x2 factorial treatments examined effects of *C. mexicana* on broilers' immune response. 200 day old Cobb chicks were assigned to the following treatments: T1 control; T2 control plus LPS or PHA; T3 control plus *C. mexicana*; T4 control plus *C. mexicana* plus LPS or PHA. For the LPS challenge; nitrites concentration, leukocyte and erythrocyte count were measured. Phytohemagglutinin (PHA) was injected into the wing web and thickness and leukocyte populations were measured at 3, 6, 12,24 and 48 h.Weight gain, feed intake and feed conversion ratio was measured. The liver, bursa, thymus, and spleen were weighted. T3 had higher ($P<0.05$) average daily gain, total weight gain; lower ($P<0.05$) feed intake and feed conversion ratio. Treatment 3 and T4 had better-feed conversion ratio compared with T1 and T2. There were differences ($P<0.05$) for thymus, bursa and spleen weights among treatments, with higher ($P<0.05$)weights for LPS treatments. For the blood cells T2 had the highest ($P<0.05$) counts of leukocyte and erythrocytes. Treatment with LPS and without *C. mexicana* had higher levels of nitrites ($P>0.05$). PHA increased wing web thickness at 3, 6, 12, 24 and 48 h post injection. The percentage of eosinophils in the wing web were smaller at 3,12,24 and 48 h post-PHA injection for treatment with *C. mexicana*. *C. mexicana* extract could be a possible alternative to antibiotic growth promoters in broilers.

Introduction

The use of antibiotics as growth promoters in broilers has provided an adequate intestinal integrity for a better digestion and absorption of nutrients. However, in the presence of low levels of antibiotic, resistant cells survive and grow, producing an antibiotic-resistant

population. It is for this reason that consumers have concerns about the safety of poultry products in many countries (Bell, 2009). Consequently, the use of antibiotics for broilers has been limited worldwide. As a result alternatives are under research; herbs, spices, and various plant extracts have received attention as possible growth promoter replacements(Hernandez *et al.*, 2004;Yang *et al.*, 2009; Salianeh *et al.*, 2011; Sadeghi *et al.*, 2012; Pickler *et al.*, 2013; Eyng *et al.*, 2015).It has been shown that the incorporation of herbs and their associated essential oils into their diet may provide beneficial effects on poultry performance and health due to the antimicrobial activity of their phytochemical components (Lee *et al.*, 2003). *Chrysactinia mexicana* Gray, commonly known as false Damiana is a small shrub distributed throughout the southwest United States and central and northern Mexico (Rzedowski and Rzedowski, 2001). The major chemical components of *C. mexicana* are eucalyptol (41.3%), piperitone (37.7%) and linalyl acetate (9.1%) (Cardenas *et al.*, 2005; Picard *et al.*, 2016; Cuevas *et al.*, 2016; Gang, 2013). Piperitone is a natural monoterpene ketone which is a component of some essential oils. Both stereoisomers, the D- form and the L-form, are known. The D-form has a peppermint-like aroma and has been isolated from the oils of plants from the genera Cymbopogon, Andropogon, and Mentha. Eucalyptol is a natural organic compound that is a colorless liquid. It is a cyclic ether and a monoterpenoid. Linalyl acetate is a naturally occurring phytochemical found in many flowers and spice plants (Gang, 2013). Cassani *et al*. (2015) demonstrated that *C. mexicana* aqueous extract induced an antidepressant effect in mice. Calzada *et al.* (2006) found that *C.mexicana* showed antiprotozoal activity against *Entamoeba histolytica* and Giardia lamblia, and also had an effect on *Trichomonas vaginalis* trophozoites (Calzada *et al.*, 2007). Alanis *et al.* (2005) studied the in vitro antimicrobial effects of *C. mexicana*, which have demonstrated some bactericidal activity. Juarez *et al.* (2010) evaluated the effect of *C. mexicana*in maize weevil (Sitophilus Zeamays Motsch) and found that the leaf powder totally prevented F1 progeny from emerging. Cardenas *et al.* (2005) found that the essential oil of leaves of *C. mexicana* completely inhibited *Aspergillus flavus* growth. Moreover, Molina *et al.* (2007) found that *C. mexicana* showed the greatest antimicrobial activity against the drug resistant strain of Mycobacterium tuberculosis. Garcia *et al*. (2016a) tested the effect of *C. mexicana* ethanolic extract on laying hens challenged with *Salmonella typhimurium*, Colony forming units (CFU) were measured in laying hens organs (gizzard, ceca, crop and duodenum) and, hens receiving the *C. mexicana* extract had similar log CFU counts as the antibiotic treatment and, *C.mexicana* extract showed a bactericide effect. In addition, Garcia *et al.* (2016b) in a field study with laying hens with Salmonella infection in the backyard found a bactericide effect with the use of *C. mexicana*. The innate immune response is the first line of defense against pathogen exposure. Pathogen exposure induces transcription and translation of genes for cytokines and other immune mediators that promote inflammatory responses and induce acute-phase protein production (Zhang *et al.*, 1995; Male *et al.*, 2006; Abbas *et al.*, 2008). The immune response to a wing-web injection of phytohemagglutinin (PHA) consists of a cutaneous basophil hypersensitivity (CBH) response. This response primarily involves lymphocytes and basophils, which infiltrate in the first 2-6 h post challenge (lymphocytes) and 24-48 h post-challenge (basophils) (Koutsos *et al.*, 2006; Koutsos *et al.*, 2007; Silva *et al.*, 2011). Nitric oxide (NO) is produced by macrophages through activation of the inducible enzyme nitric oxide synthase,

which has powerful antiviral and anticancer properties (Stuehr and Nathan, 1989; Hussain and Qureshi, 1997; Alderton *et al.,* 2001). Lypopolysaccharide (LPS) has been found to be a potent activator of immune and inflammatory cells, resulting in various pro-inflammatory cytokines (Dil and Qureshi, 2002). LPS challenge has been found to increaseNO secretion(Li *et al.* 2015).The objective of the present study was to evaluate the effect ofthe anti-inflammatory effects of *C.mexicana* Gray extract in growing chicks challenged with either LPS or PHA.

Materials and Methods

Plant extract

Plant collection was made from Guadalcazar village, located in a semi-desert area in the center zone of Mexico. Leaves were separated from the plants, placed on plates and dried for three weeks at room temperature. The leaves were then ground, and the extract was obtained by common extract methods, such as heat extraction, gravity column or percolation technique with ethanol. Two hundred g of ground leaf powder samples were placed in a column by gravity or percolation and the solvent was added. This sat for 48 h, using about 5 L of ethanolsolvent, the samples were then dried in an extraction chamber. The obtained extract was then concentrated at reduced pressure to 29 °C with a rotavapor(R-210/R-215 Buchi) (NCCLS, 2012; Pelczar and Reid,1958;Sidney *et al.,* 1978). Finally, the extract was dried by the freeze drying process (cryodesicattion).

In vivo

Designs for the following experiments were similar, and exceptions are noted below. Two experiments were designed with a 2 x 2 factorial arrangement of treatments; experiment one consisted of two *C. mexicana* levels and two levels of LPS. 100 d old chicks (Cobb strain) were randomly assigned to one of 20 pens (n=5/pen). At 14 d, chicks within pens were randomly assigned to one of two LPS treatments: chicks were either not injected (control) or were injected with 2 mg LPS/kg body weight (BW) intra-abdominally. At six h post-LPS injection, four chicks/pen were bled via cardiac puncture into heparinized tubes for plasma isolation. The *C. mexicana* extract was administered orally via an esophageal cannula during 15 days at 20 mg/ml. Feed and water was offered ad libitum. Feed was formulated to meet or exceed the National Research Council (NRC, 1994) requirements for growing chicks (Table 1). All chicks were housed in identical brooder battery cages (Petersime Inc., Gettysburg, OH) in a temperature controlled room (25 °C) under 24 h light. The dependent variables measured included: initial body weight, weight gain, final weight, feed intake and plasma NO at six h post-LPS. The liver, bursa, thymus, and spleen were removed and weighted. In addition, leukocyte and erythrocyte counts were determined by Natt and Herrick´s Stain method. Briefly, a standard red blood cell diluting pipette was used to dilute whole anticoagulated blood with the Natt& Herrick´s solution at the rate of 1:200. The diluted blood was allowed to mix for two minutes before it was discharged into the hemacytometer counting chamber. Then using the high dry (40X) objective of the microscope, the total number of red and white cells were counted (Campbell, 1995). The concentration of the NO metabolite was measured in plasma using the Griess reagent method. Nitrite assay. Nitrite levels were measured in 96-well microtiter plates by mixing 100 uL of the macrophage culture supernatant with an equal volume of Griess reagent (one part 0.1% naphthylethylene-diaminedihydrochloride to one part 1% sulfanilamide in 5% phosphoric acid) as described by Hussain and Qureshi (1997).

After 10 min incubation at room temperature, the change in color indicative of nitrite presence was quantified by reading the plates at A540 on an ELISA plate reader (Difco Laboratories, Inc., Detroit, MI 48232). An average of three measurements per sample was used in final analysis. A standard curve of optical densities (OD) at A540 was generated using various concentrations of sodium nitrate dissolved in RPMI-1640 growth medium (without phenol red). Nitrite levels of the culture supernatants were determined by comparing their OD values with that of the standard.

Experiment two consisted of two *C. mexicana* levels and two levels of PHA. 100 d old chicks (Cobb strain) were randomly assigned to one of 20 pens (n=5/pen). The *C. mexicana* extract was administered orally via an esophageal cannula over 15 days at 20 mg/ml. Feed and water was offered ad libitum. Feed was formulated to meet or exceed the National Research Council (NRC,1994) requirements for growing chicks (Table 1). All chicks were housed in identical brooder battery cages (Petersime Inc., Gettysburg, OH) in a temperature controlled room (25 °C) under 24 h light. At 14 d all birds were injected into the wing web with PHA (L8754, Sigma Aldrich, St. Louis, MO, USA; 100 µg in 100 µl PBS). CBH response was examined by measuring wing web thickness in the injected wing and in the uninjected wing with low pressure digital calipers (model 573, Mitutoyo Inc., Kawasaki, Japan) at 3, 6, 12, 24, and 48 h post-injection (n=2/pen*time period). CBH response was determined based upon the difference in the skin thickness between the injected and the uninjected wing. Tissue biopsies (n=2/pen*period time) were taken just after thickness measurements, using a dermal biopsy punch (3 mm biopsy punch, #33-32, Miltex Inc., West Sacramento, CA, USA). Cell infiltration was quantified by microscopic evaluation of tissue sections (Nikon Photo Shoot microscope). At least 100 leukocytes were counted at the site where the swelling was maximal, and leukocyte infiltration was most dense. Data are presented as the percentage of each cell type present within the tissue section.

Data Analysis

For the statistical analysis, a complete randomized design was used to assess the extract activity. Analysis of variance was performed with PROC GLM of SAS, and Tukey means with the statistical analysis system (SAS,1991) software program.

Results and Discussion

Performance parameters were affected by *C. mexicana* inclusion; T3 had higher ($P<0.05$) average daily gain, total weight gain; lower ($P<0.05$) feed intake and feed conversion ratio than the other treatments (Table 3). Treatment 3 and T4 that had *C. mexicana* inclusion had better feed conversion ratios compared with T1 and T2. In the case of thymus, bursa, and spleen weights there were differences ($P<0.05$)for treatments challenged with LPS showing higher ($P<0.05$) thymus, bursa and spleen weights than treatments without LPS challenge. For the blood cells; T2 had the highest ($P<0.05$) counts of leukocyte and erythrocytes in comparison with the other treatments (Table 2).

Plasma NO was increased by LPS treatment after six h post injection. Treatment injected with LPS and without *C. mexicana* had higher levels of nitrites ($P<0.05$) than treatment injected with *C. mexicana* (Figure 1). Nitrites concentration in the group without *C. mexicana* was almost two times higherthan the group with *C. mexicana* extract.

PHA injection increased wing web thickness at 3, 6, 12, 24 and 48 h postinjection. Figure 2

shows that treatment with PHA and without *C. mexicana* extract had higher (P<0.05) values than treatment with PHA and with *C. mexicana* extract. Levels of inflammation remained high at 24 and 48 h in the treatment without *C. mexicana*. While the inflammation decreased (P<0.05) at 24 and 48 h with the *C. mexicana* extract (Figure 2).

The percentage of eosinophils in the wing web was affected by time (P<0.05) and was greatest at 12 h post-PHA for treatment – *C.mexicana* + PHA (P<0.05) (Figure 3. A). Additionally a significant interaction (P<0.001) between PHA x *C. mexicana* x time demonstrates that chicks with *C. mexicana* and PHA challenge had lower percentage of eosinophils than treatment without *C. mexicana* at 3, 12, 24 and 48 h. Treatments without PHA challenge had lower (P<0.05) eosinophils percentage values than treatments challenged with PHA (Figure 3. A). The percentage of heterophils was affected by time (P<0.05) and was greatest at six h post-PHA for treatment – *C. mexicana* + PHA (P<0.05) (Figure 3. B).Aftersix h the percentage declined over time. Treatment + *C. mexicana* + PHA had reduced (P<0.05) heterophils percentage values at 12 and 24 h. Treatments without PHA challenge had lower (P<0.05) heterophils percentage values than treatments challenged with PHA (Figure 3. B). The percentage of basophils was affected by time (P<0.05) and was greatest at 6 h post-PHA for treatment +*C. mexicana* + PHA (P<0.05) (Figure 3. C). An interaction between *C. mexicana* x time (P<0.001) demonstrates that chicks with *C. mexicana* had lower basophil percentage at 48 h post injection as compared to chicks without *C. mexicana*. Treatment -*C. mexicana* + PHA had reduced (P<0.05) basophils percentage values at 24 h post injection. Treatments without PHA challenge had lower (P<0.05) basophils percentage values than treatments challenged with PHA(Figure 3. C).

Performance parameters were affected by *C. mexicana* inclusion. Treatment Control +*C. mexicana* had higher average daily weight gain, total weight gain; and showed a better feed conversion ratio than the other treatments. These results agree with those found by Garcia *et al.* (2016b) in a study with Plymouth Rock Barred pullets using *C. mexicana* which found better average daily weight gain and a better feed conversion ratio. In the case of thymus, bursa and spleen weights there were significant differences for the treatments with LPS challenge, showing higher organ weights than treatments without LPS challenge. These results are in agreement with Koutsos *et al*. (2006) that found an increase in bursa, thymus, and spleen weights after an LPS injection in chickens. For the blood cells; treatment with PHA had the highest counts of leukocyte and erythrocytes in comparison with the other treatments. There are no previous studies about the effect of *C. mexicana* extract on the wing web inflammation induced with PHA or serum nitric oxide (NO) production induced by LPS. During the first two weeks of age, chicks are very sensitive to infections and mortality is high during this period. Therefore, feed additives that help to enhance the chicks' immune response are highly recommended. It has been reported that *C. mexicana* has some bactericide activity in laying hens and pullets challenged with *Salmonella typhimurium* (García *et al.*, 2016ab). Nitric oxide (NO) is an important host defense effect or in innate immunity (Male *et al.*, 2006; Abbas *et al.*, 2008). It is generated by various tissues from L-arginine (Stuehr and Nathan, 1989). The increase of NO concentrations in this study induced by lipopolysaccharide (LPS) were suppressed significantly by *C. mexicana* extract. Li *et al*. (2009)found that dietary supplementation with chitosan (a natural alkaline polysaccharide) enhanced immune functions which were associated with the increase of NO secretion in the small intestine

in broilers. Li *et al*. (2015) found that the increase of nitric oxide concentrations induced by LPS was reduced by chitosan in weaned piglets. It was reported that chitosan inhibits the production of NO in LPS activated RAW 264.7 cells, but stimulates the release of NO in resting or unstimulated peritoneal macrophages (Chou *et al.*, 2003).

PHA injection increased wing web inflammation at 3, 6, 12, 24 and 48 h post-injection. Treatment with *C. mexicana* had lower inflammation values than treatment without *C. mexicana*. Silva *et al*. (2011) tested the effect of different levels on Vitamin E and found a lasting cell mediated response with 65mg/kg when chickens were challenged with PHA. Koutsos *et al*. (2007) studied the effect of lutein on cutaneous basophil response in chickens and found cell reactions up to 48 h post PHA-injection. The percentage of eosinophils, heterophils and basophils in the wing web was affected by time and, in general, treatment with *C. mexicana* showed less percentages than treatments without *C. mexicana* in chicks challenged with PHA.

Table.1 Basal diet composition1

Ingredient	g/kg diet
Yellow corn 8%	541.8
Soybean meal 46.5%	369.5
Calcium carbonate 38%	5.6
Cornstarch	20.0
Vegetable oil	30.2
Dicalcium phosphate	17.5
Sodium chloride	5.0
Vitamin mix[2]	2.5
Mineral mix[2]	2.5
DL-Methionine	3.5
Threonine	0.5
L-Lysine	0.4
Choline	1.0
Chemical composition	
Metabolisable energy, Kcal/kg	2954
Crude protein, %	23.91
Calcium, %	0.42
Phosphorus,%	0.55
Methionine, %	0.64
Lysine, %	1.27
Threonine, %	0.88

[1]Diet was offered ad libitum for the duration of the trial, and was formulated to meet or exceed all requirements for growing chickens (NRC,1994).

[2]Vitamin mix provided (per kg final diet): thiamin, 1.8 mg; riboflavin, 3.6 mg; pantothenic acid, 11.5 mg; niacin, 35 mg; pyridoxine, 3.5 g; folic acid, 0.6 mg; biotin, 0.2 mg; vitamin B-12, 10 µg; retinylpalmitate, 0.9 mg; cholecalciferol, 50 µg, all-rac-α-tocopheryl acetate, 36.8 mg; menaquinone, 5 mg. Mineral mix provided (per kg final diet) selenium, 0.2 mg; copper, 8.1 mg; zinc, 40.7 mg; manganese, 62 mg; iron, 105.4 mg; iodine, 0.35mg.

Table.2 Means of broiler performance, organ weights and blood cells with different treatments

Treatment	T1	T2	T3	T4	SEM
Performance					
Initial body weight [g]	40.95	42.17	41.05	41.36	0.048
Final bodyweight [g]	449.05[b]	439.92[b]	501.75[a]	453.98[b]	2.145
Total weight gain [g]	408.10[b]	397.75[b]	460.70[a]	412.62[b]	4.135
Average daily gain [g]	29.15[b]	28.41[b]	32.90[a]	29.47[b]	0.211
Feed intake [g]	496.23	494.45	492.78	489.21	5.210
Feed Conversion Ratio	1.21[b]	1.24[b]	1.06[a]	1.18[b]	0.012
Organ weights					
Thymus[g]	0.829[b]	0.878[a]	0.833[b]	0.861[a]	0.024
Bursa [g]	0.918[b]	0.976[a]	0.922[b]	0.991[a]	0.034
Spleen [g]	0.301[b]	0.372[a]	0.314[b]	0.368[a]	0.019
Blood Cells					
Leukocyte/ mm3	1.78[b]	2.74[a]	1.80[b]	1.85[b]	0.011
Erythrocytes/mm3	26.12[b]	45.91[a]	27.52[b]	28.27[b]	3.221

[a,b,c,d]Means within columns with different letter are significant different (P<0.05). T1=Control basal diet; T2=Control +LPS or PHA, T3=Control + *C. mexicana*; T4= control + *C. mexicana* + LPS or PHA.

Fig.1 Means of nitrites concentration of chick serum of different treatments

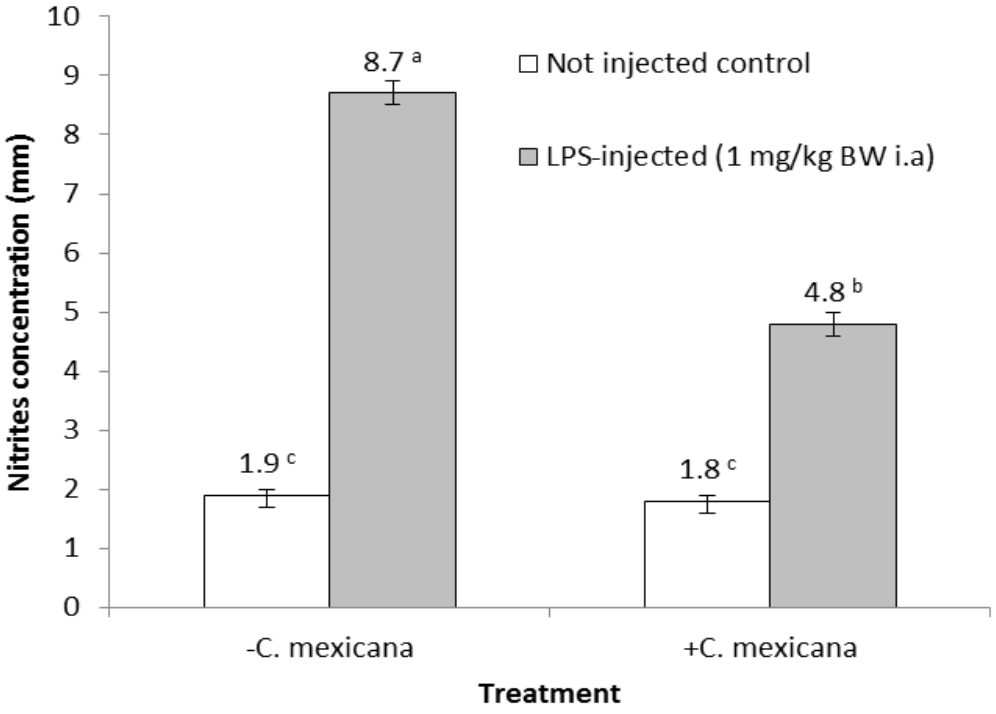


Fig.2 Means of inflammation (mm) in the wing-web at different time after PHA injection

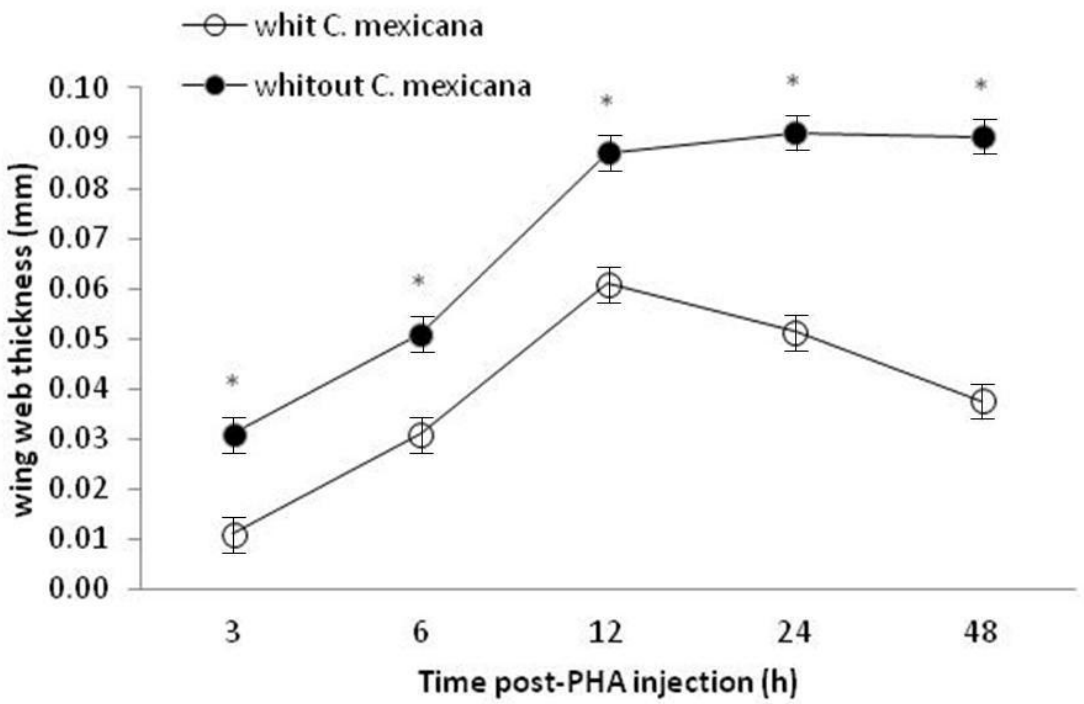
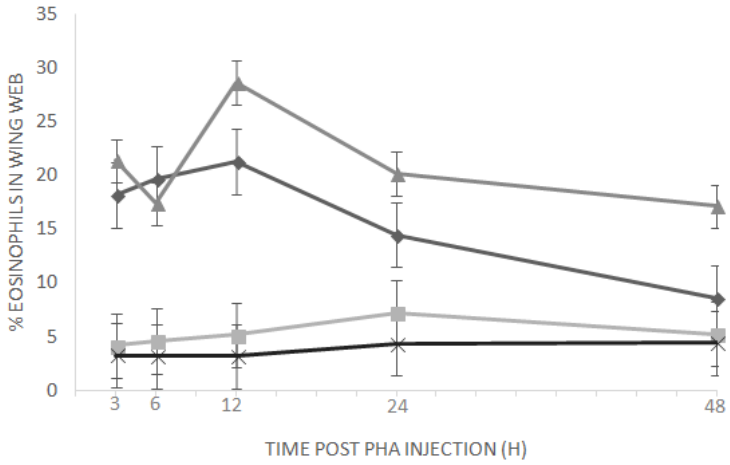
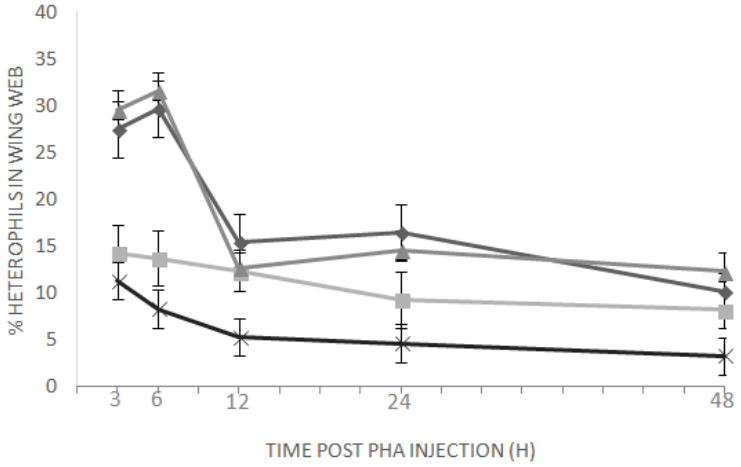


Fig.3 Effect of PHA injection on leukocyte populations in the wing web of chickens with *C. mexicana* and without *C. mexicana*. A. Eosinophils percentages. B. Heterophils percentage. C Basophils percentage

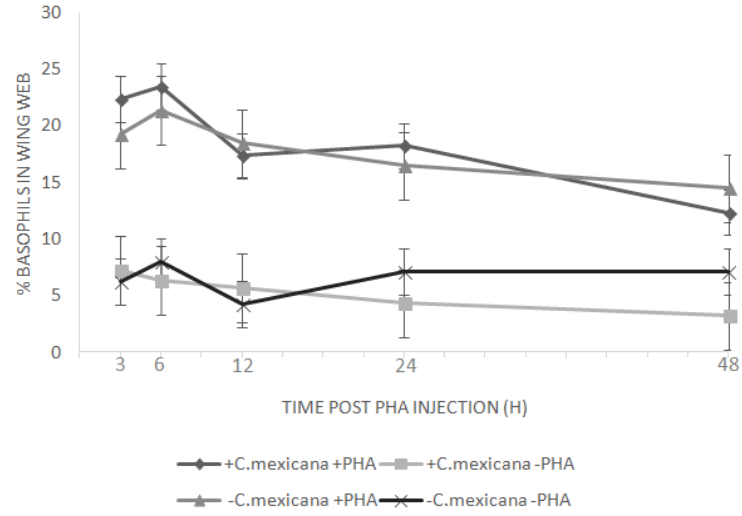
A



B



C



Treatments without PHA challenge had lower leukocyte percentages values than treatments challenged with PHA. Koutsos *et al.*, (2007) measured cell infiltration testing different levels of lutein and found that basophil infiltration was greater for chicks fed 0 mg of lutein after a PHA challenge. Although the *C. mexicana* extract mechanism of action is not clear, *C. mexicana* may contribute macrophages to produce less NO, helping the chicks immune system to counteract the PHA inflammation.Some of the research findings for *C. mexicana* that may contribute to enhance immunity were performed by Molina *et al*. (2007) that tested three different extracts of *C. mexicana* (aqueous, methanol and diethyl ether) and found a bactericide effect with two strains of Mycobacterium tuberculosis. Moreover, Cantrell *et al*. (1998) reported *C. mexicana* to have antimycobacterial activity. In another experiment, Delgado and Rios (1991) performed a phytochemical analysis and monoterpenes were identified. Another experiment with essential oil from leaves showed some antifungal activity (Cardenas *et al.*, 2005). The pharmacological effect of *C. mexicana* could be related to intracellular concentration regulation of Ca^{++} (Park *et al.*, 2011). The action mechanism of a majority of *C. mexicana* components has been reported as eucalyptol inhibited contractions induced by

carbachol (Shah *et al.,* 2011;Mangprayool *et al.,* 2013). It also has anti-diarrheic activity (Yvon *et al.,* 2012). Eucalyptol and 26 other diterpenes have been reported to decrease cytokines IL-2 (Th1) and IL-10 (Th2) that are anti-inflammatory inhibiting the response of T cells (Ku and Lin, 2013). Finally, it has been reported that the essential oil from *Cymbopogon proximus* contains piperitone as the highest compound (73.8%). This compound antagonizes the actions of serotonin and histamine, by interaction of its receptors (Al-Taweel *et al.,*2013). All the above experiments showed that *C. mexicana* might enhance the immune response in growing chicks. According to the results of this experiment, the use of *C. mexicana* extract could be a good alternative for antibiotic growth promoters in broilers.

In conclusion the *Chrysactinia mexicana* extract showed good performance traits. The use of the extract decreased the NO secreted levels and had a lower cell infiltration in chicks challenged with LPS and PHA. More studies are warranted to elucidate the mechanism of action of the *C. mexicana* extract.

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