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Original Article

Phytobiotic Activity of Piper Auritum and Ocimum Basilicum on Avian E. Coli

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ABSTRACT

Natural antimicrobials, known as phytobiotics, are used in bacterial infections. The objective of this study was to evaluatethe phytobiotic activity, *in vitro* and *in vivo*, of an extract and an essential oil of *Piper auritum* and *Ocimum basilicum* on avian *Eschericia coli* serotype O2 in broiler chickens experimentally infected. For the *in vitro* test, extracts at 4, 8, 12 and 16% in water-based solvent or alcohol at 70% were prepared from leaves of both plants. In the essential oils, solvents at 10% were used. A concentration of 1×10^8 CFU mL^{-1} of bacteria was seeded and on each sense disc, 25 µL of the extract or essential oil were poured, except on the positive or negative control. The diameter of the inhibition zone (DIZ) of bacterial growth was measured. In the *in vivo* test, 40 chickens were inoculated, by intratracheal route, with a bacterial suspension of 1×10^8 CFU mL^{-1}. *E. col* was identified and isolated from the organs; mortality, morbidity and relative weight of the organs were measured, and postmortem lesions and histopathologic findings were observed. A completely randomized design and the Kruskal-Wallis test for data analysis was used. By increasing the concentration of the extract, DIZ was greater; at the time of slaughter, differences in body weight ($p<0.05$) were found and the majority of lesions were observed in lungs. It is concluded that leave extracts of *P. auritum* and *O. basilicum* had phytobiotic activity on *E. col* serotype O2.

INTRODUCTION

It is calculated that about 100,000 plants produce secondary metabolites (Dixon, 2004) and the biological activity of some of them is well known (Gurib-Fakim, 2006). These plants are used without restriction, in dose or age of the animal, in the form of extracts or essential oils, as an alternative to growth promotive antimicrobials (AGP), due to their capacity to elevate digestive enzyme activity (Adebolu & Abiola, 2005), nutrient absorption, increase in food intake (Chávez *et al*., 2015), reduction in undesirable bacterial population (Acosta *et al.,* 2003), decreased intestinal activity associated with lymphatic system and increased precaecal digestion of nutrients; generally reflecting greater intestinal flora balance (Witte, 2000; Windisch *et al*., 2008).

Some plants or part of them contain natural antimicrobials that are known as phytobiotics, these types of antimicrobials are considered as potentially safe sources (Griggs & Jacob, 2005; Rodríguez, 2011). This activity has been associated with secondary metabolites, such as: carvacrol, thymol, eugenol, safrole, perillaldehyde, cinnamaldehyde, α terpineol, linalool and cinnamic acid (Lataoui & Tantaoui-Elaraki, 1994; Consentino *et al*., 1999; Burt, 2004; Burt *et al*., 2005). Other reported components are borneol, γ-terpinene and *p* cymene that have a synergic or antagonic activity (Consentino *et al*., 1999; Vardar-Ünlü *et al*., 2003).

Escherichia coli is a normal habitant of the intestine of birds and can remain for a long time in poultry farms (Cortes, 2008; García-Compean *et al*., 2011) without causing any adverse effect, since it generally responds to a secondary infection subsequent to a *Mycoplasma* sp first attack and particularly by the infectious bronchitis virus, which is a predisposing factor of colibacillosis (Cook *et al*., 1991; Nakamura *et al*., 1999) or even micro environmental due to high concentrations of ammoniac, CO_2 or dust; the latter damages the cilia of the respiratory tract, causing colibacillosis, the main cause of condemned carcasses and considered as potential public health problem (Dozois *et al.*, 2000; Mellata *et al.*, 2003; García-Compean *et al.*, 2011).

Among *E. coli* properties there is the belonging of specific serotypes such as: 01:K1, 02:K1, 35 and O78:K80 (Dho-Moulin & Fairbrother, 1999; Ewers *et al.*, 2003) being serogroups O2 and O78 responsible for 80% of the infections caused by colibacillosis in the field (Dho-Moulin & Fairbrother, 1999; Horne *et al*., 2000; Ewers *et al.*, 2003; Blanc *et al.*, 2007). Up to date, bacterial resistance to antimicrobials is already known, due to its uncontrolled use (Kalra, 1998; Kalemba & Kunicka, 2003).

The genus *Piper* contains approximately 1500 species, with nearly 1000 species in tropical America. It has been widely studied for being an important source of bioactive components, with antimicrobial properties for human, plants and animals (Dorman & Deans, 2000; Kato & Furlan, 2007; Regasini *et al.*, 2009; Monzote *et al.*, 2010).

Piper auritum belongs to Piperaceae family. It is native to Mexico and is widely distributed in tropical America (Roig, 1988). Valsara (1994) and Oudhia (2003) demonstrated that this plant has antiseptic, antifungic and antioxidant effect, the latter is very near to the reported for α-tocopherol (Ramya *et al.*, 2010). The chemical analysis of the essential oil of *Piper auritum* reveals that safrole is its main component, occupying about 70 to 90% (Gupta *et al*., 1985; García *et al*., 2007; Sánchez *et al*., 2009). Besides safrole, the essential oil of *Piper auritum* contains about 40 substances in lower amount, such as: thymol, carvacrol, myristicin, linalool, borneol, camphor, cineol, methyl eugenol and a wide variety of benzene components (Domínguez *et al*., 1962; Oliveira *et al*., 2004).

The genus *Ocimum L.* is formed by about 30 species, 16 of which are native to Africa. Its distribution area goes from tropics to subtropics of America and Europe (Mahabir, 1995). It has antimicrobial and spasmolytic properties (Acosta *et al*., 2003; Ramya *et al*., 2010). The reported active components of *O. basilicum, basilicum* variety are: linalool (54.28 %), 4- allylanisole (26.50%), eugenol (9.54%), 1.8 cineol (4.21%) and for purpurascens variety are: E-methyl cinnamate (55.95%), linalool (21.30%), Z-methyl cinnamate (16.85%) and 1.8 cineol (1.44%), besides having phenolic acids derived from cinnamate acid and flavonoids (Acosta *et al*., 2003). Roldán *et al*. (2010) indicate that the largest components of *O. basilicum* are: β-linalool (46.67%) and estragole (27.43%).

The objective of this study was to evaluate the phytobiotic activity of the extract and essential oil of *Piper auritum* and *Ocimum basilicum*, first *in vitro* determining the diameter of the inhibition zone of bacterial growth and *in vivo,* in the productive performance, bacterial growth, relative weight of organs and histopathologic findings in broiler chickens experimentally infected with avian *Escherichia coli* serotype O2.

MATERIAL AND METHODS

Collection of samples and storage.

The leaves of *Piper auritum* and *Ocimum basilicum* were recollected in the Unidad de Producción e Investigación Agrícola y Pecuaria of the Instituto Tecnológico de Conkal. The leaves were dried in a forced air oven at 60°C for 24 hours; subsequently, they were milled in order to obtain flour from the leaves using a sieve size of 1 mM thickness. The experiment consisted of two tests, the first was *in vitro* and the second *in vivo*.

In vitro test

Preparation of aqueous extract

Four, eight, twelve and sixteen grams of flour from leaves of each plant were weighed, submerged in 100 mL of boiling distilled water and underwent decoction for 15 minutes, and then was filtered using a cloth of linen (López-Casamayor, 2007). Once the extract was cooled it was filtered again using Whatman paper number 2 (López-Casamayor, 2007).

Preparation of alcoholic extract

Four, eight, twelve and sixteen grams of flour from the leaves of each plant were weighed and submerged in 100 mL of alcohol at 70%. The solutions were agitated using a hot plate stirrer (IKA, C-MAG-HS7, North Carolina, USA) for 15 minutes and allowed to sit for 48 hours at room temperature. Extracts were first filtered using a cloth of linen (López-Casamayor, 2007), followed by centrifugation at 7000 rpm for 10 minutes

using a centrifuge (Beckman TJ-6, Illinois, USA); the supernatant was filtered using a Whatman paper number 2 (Mamoru *et al*., 1996; Tequida-Meneses *et al.,* 2002). Both extracts were poured into amber glass bottles and stored in the refrigerator. At the time of use they were sterilized by filtration using Millipore membrane of 0.8 to 0.2 µM (López- Casamayor, 2007).

Obtaining essential oils

Forty grams of flour from the leaves of each plant were weighed, submerged in 200 mL of distilled water or 200 mL of alcohol at 70% and allowed to sit for 48 hours at room temperature. Both solutions were subjected to steam stripping using a rotavapor (Büchi, R-114, Switzerland), at a temperature of 70°C and at 40 rpm (Sánchez-Castellanos, 2006). Oils obtained were poured into amber glass bottles and stored in the refrigeration at 4°C until further usage (Sánchez-Castellanos, 2006).

Inoculation of Petri dishes

Petri dishes with eosin methylene blue agar were inoculated with 1× 10^8 CFU mL^{-1} of *E. coli* serotype O2 in the four directions using a swab. The correct density of the inoculum was performed using the method described by Sánchez *et al.* (2009). The Sensi-Discs had a diameter of 6 mM and were made with Whatman paper number 2 and were distributed on the agar at no less than 22 mM from each other and 14 mM from the border of the Petri dish. Negative controls of distilled water and alcohol at 70% were used for the extracts; petroleum ether was used in the essential oils. Positive controls were also used for essential extracts and oils, which consisted of commercial Sensi-Discs (Whatman, Sigma-Aldrich, Mexico) loaded with Enrotrim at 10% (enrofloxacin and trimethoprim), Macromycin E (colistin sulphate, erythromycin thocyanate and ethylenediamine dehydroiodide) and Doxy20 (doxycycline at 20%). On each Sensi-Disc, 25 µL of extract (aqueous or alcohol at 70%) or essential oil were poured, except on negative or positive control. The inoculated Petri dishes were incubated at 35°C for 18 hours for subsequent measurement.

Phytobiotic activity measurement

Agar diffusion method, described by Kirby-Bauer (Bauer *et al.,* 1966), was used for measuring antimicrobial activity by presence or absence of the diameter in the inhibition zone (DIZ) of bacterial growth. The diameters were measured on the back of the Petri dish, placed against a brilliant light, using a millimeter rule in angles of 120°. The average of three measures was reported (Koneman *et al.,* 1991).

In vivo test

Preparation of the strain

E. coli strain was cultured on eosin methylene blue agar at 37°C for 24 hours. The obtained culture was centrifuged at 3,400×g for 15 minutes; subsequently, it was washed and resuspended in PBS (pH 7.4). Bacterial concentration was measured by spectrophotometer at 660 mM and an absorbance of 0.8, using a plate reader (Thermo Scientific, Multiskan GO, Germany).

Ethical Considerations of the Study

The management and care of the chickens were in accordance with Animal Research Ethics and following guidelines approved in official techniques of animal care and health in Mexico (Ley Federal de Sanidad Animal; articles 19 to 22), NOM-033-ZOO-1995: Humanitarian slaughter of domestic and wild animals, and the international guiding principles for biomedical research involving animals by the Council for International Organizations of Medical Sciences (CIOMS).

Experimental animals

A total of 50 Ross 308 male chickens, 35 days old, without vaccines, coming from a clean and safe zone, were distributed in five treatments with ten replicates each one (Table 1). Each treatment was located in 5 experimental pens of approximately 1.95 m^2, each equipped with a gallon type waterer (JAT, Jalisco, México). The negative control group was located in a distant pen and isolated from the experimental shed equipped with sanitary rugs to avoid cross-contamination.

Table 1 – Distribution of chickens inoculated with avian *E. coli* serotype O2 and treated with extracts of leaves with phytobiotic properties.

Group	Treatment	Dose	Inoculated (*E. coli*)
1	*Piper auritum*	30 mL of extract at 10%	Yes
2	*Ocimum basilicum*	30 mL of extract at 10%	Yes
3	Control	Without extract and without antimicrobial	Yes
4	Control	Enrofloxacin at 10%	Yes
5	Control*		No

*Negative control.

Chicken inoculation

After a period of adaptation of seven days in the experimental pens, chickens were inoculated with 0.5 mL of a bacterial suspension of 1×10^8 CFU mL^{-1} of *E. coli* serotype O2 by intratracheal route, using 1 mL syringe (Kwaga *et al.,* 1994; Yunis *et al.*, 2002; Rawiwet & Chansiripornchai, 2009).

Extract administration

Thirty mL of aqueous extract was orally administered to each chicken 36 hours post inoculation, using a 1 mL syringe without needle (Plastipak, México); subsequently, it was administered every morning until 24 hours before being humanely slaughtered.

Evaluated variables

Chickens were fed a diet based on soybean–sorghum, free of antibiotics, fungicides and coccidiostats. Body weights were recorded at the beginning (36 days), at the moment of inoculation (42 days) and at the end of the trial (59 days). At the end of the trial, chickens were humanely slaughtered according to the Mexican Official Standard NOM 033-ZOO-1995. Immediately, bursa of Fabricious, liver, heart, kidney, lung, spleen and gastrointestinal track were weighed. Weights were recorded as relative percentage of body weight (Huff *et al.,* 2006).

Mortality and morbidity were measured as: 1) mortality: for chickens who died during the first nine days postinoculation and showed postmortem lesions of colibacillosis; 2) morbidity: for chickens that survived inoculation until the trial ended and showed lesions of colibacillosis at necropsy (Rawiwet & Chansiripornchai, 2009). The integrity of air sacs, lung, pericardium and liver was observed and histopathologic findings of only lung and liver were described (Kleven *et al.*, 1972; Charleston *et al.,* 1998).

Isolation, identification and quantification of E. coli

Samples (10 g) of lung, liver, heart and spleen were taken and ground according to the technique described by Willis *et al*. (2008). The pool was diluted in serials (1:10) in 0.1% of saline solution. Subsequently, 100 µL of each dilution was placed on Agar McConkey and incubated at 37°C for 24 hours for its subsequent bacterial quantification as CFU g^{-1} transformed to Log base 10 (Mitsche *et al.,* 2004; Willis *et al*., 2008).

Statistical analysis

A completely randomized design was used to analyze the variables of the diameter of the inhibition zone of bacterial growth of five treatments. One chicken was considered as experimental unit and the percentages of relative weights of the organs were analyzed using Kruskal-Wallis test, previous transformation of percentual data to square root of arc sine (Dughetti & De Carli, 1999). Only mortality was described. The differences between means were analyzed using Tukey test, considering significant an alpha of 0.05 (Mendahall, 1994). Data were analyzed using the statistical program SPSS v 15.0 for Windows.

RESULTS AND DISCUSSION

The aqueous extracts of *P. auritum* and *O. basilicum* had less bactericide effect than their alcoholic extracts at 4% levels. The aqueous extracts had a DIZ of 9.6 and 9.0 mM while, in the alcoholic extracts, DIZ were of 16.4 mM and 13.9 mM for *P. auritum* and *O. basilicum*, respectively (Table 2).

The obtained results show that both plants inhibit *E. coli* growth; however, while using alcohol at 70% as solvent in the preparation of alcoholic extracts, DIZ increased due to high polarity of the alcohol that allows to obtain an extract whose chemical composition contains the greater part of the components of the plant, polar or not polar; allowing to obtain an extract with greater quantity of secondary metabolites with phytobiotic activity (Sharapin, 2000), compared to the low solubility of water before the hydrocarbon skeleton of some phytobiotic components found in aqueous extracts (Kalemba & Kunicka, 2003). Authors such as Hernández *et al.* (2003), Adebolu & Abiola,

Table 2 – Diameters of the inhibition zone of bacterial growth (mM)[1] of avian *E. coli* serotype O2, challenged with extracts of leaves with phytobiotic properties.

	Aqueous Extract		Alcoholic Extract		Negative Control		Positive Control		
	Pa[2]	Ob	Pa	Ob	Ad[3]	Al	EN	DX	MM
4%	$9.6^{b}\pm0.8$	$9.0^{b}\pm0.7$	$16.4^{fg}\pm0.9$	$13.9^{ef}\pm1.7$	$6.3^{a}\pm0.4$	$10.1^{bc}\pm1.0$	$32.0^{k}\pm1.2$	$24.6^{j}\pm1.2$	$30.2^{k}\pm2.4$
8%	$10.8^{bcd}\pm0.8$	$10.7^{bc}\pm0.7$	$20.5^{hi}\pm1.1$	$16.5^{fg}\pm2.0$					
12%	$11.7^{bcde}\pm0.7$	$11.9^{bcde}\pm0.4$	$21.5^{hi}\pm1.6$	$19.0^{gh}\pm1.4$					
16%	$13.1^{de}\pm0.9$	$12.5^{cde}\pm0.8$	$22.0^{ij}\pm2.5$	$22.1^{ij}\pm1.4$					

[abcdefghijk] Different literals in column indicate difference ($p>0.05$). [1] Values are represented as mean ±S.D. [2]Plant from which the extract was obtained, Pa: *P. auritum*, Ob: *O. basilicum*. [3]Control: Negative, Ad: Pure distilled water, Al: Alcohol at 70%; Positive, EN: Enrotrim 10%, DX: Doxi20, MM: Macromycin.

(2005) and Nwinyi-Obinna *et al.* (2009) report that the genera studied are potential source of components with phytobiotic properties, mainly against pathogenic bacteria such as *E. coli*, *Salmonella typhimurium* and *Pseudomona aeruginosa.*

The percentage of the extract is an important factor in phytobiotic activity in both plants, by

increasing concentrations DIZ was greater, due to greater quantity of bioactive components. Burt *et al.* (2007) report that *E. coli* O157:H7 bacterial cell decreased gradually by increasing the concentration of carvacrol and *p*-cymene in the culture medium; both essential oils inhibited the synthesis of flagella; therefore, no more flagellated forms were observed.

The results of DIZ presented by the essential oils of *P. auritum* and *O. basilicum* are shown in Table 3. Smaller DIZ were obtained by petroleum ether (6.1 mM), which means that *E. coli* growth was not affected, while greater DIZ were for the antimicrobials Enrotrim at 10% and Macromycin with 29.6 and 29.1 mM, respectively. Phytobiotic activity of essential oils of both plants were similar ($p>0.05$). The DIZ were 20.2 and 20.09 for *P. auritum* and *O. basilicum*, respectively. Such activity coincides with Adebolu and Adiola (2005) and Reshmi *et al.* (2010), who report that essential oils of *Piper* and *Ocimum* gender present phytobiotic properties before several microorganisms such as *S. aureus, E. coli* and *S. typhi.*

Table 3 – Diameter of the zone of inhibition of bacterial growth (mM)[1] of avian *E. coli* serotype O2, challenged with essential oils of leaves with phytobiotic properties.

Plant		Control			
Piper auritum	*Ocimum basilicum*	EP[2]	EN[3]	DX	MM
20.2[b] ±3.9	20.09[b] ±1.9	6.1[a] ±0.2	29.6[c] ±3.1	23.6[b] ±2.3	29.1[c] ±4.2

[abc]Different literals in the same row indicate difference ($p<0.05$). [1]Values represent the mean ±S.D. [2]Negative control, petroleum ether 25 µL. [3]Positive control, EN: Enrotrim, DX: Doxi20, MM: Macromicine.

At the beginning of the experiment and at the moment of inoculation, the birds had a similar weight ($p>0.05$); however, at slaughter these were different ($p<0.05$) (Table 4). There was no difference ($p>0.05$) between the highest body weight recorded on chickens of group 1, inoculated and treated with *P. auritum* extract and chickens of group 4, inoculated and treated with Enrofloxacin at 10%. Chickens inoculated and treated with *O. basilicum* had similar body weight ($p>0.05$) to those of negative control (group 5), but lower than the group of *P. auritum* extracts ($p>0.05$). The difference in body weights recorded was due to chickens inoculated with *E. coli,* because of severe anorexia mainly caused by lesions in organs such as: liver, spleen, kidney and digestive system (Dunnington *et al.,* 1991; Gomis *et al.,* 1997; Huff *et al.,* 2006).

Table 4 – Body weight of chicken inoculated with avian *E. coli* serotype O2, treated with extracts of leaves with phytobiotic properties.

Treatments[1]	Body weight[2]		
	Initial weight	Inoculation weight	Weight at slaughter
Group 1	2118.30 ±110.29	2703.00 ±148.34	3439.00[a] ±157.33
Group 2	2081.40 ±109.35	2748.50 ±131.61	3390.50[ab] ±176.89
Group 3	2126.50 ±143.90	2630.50 ±294.62	3160.00[b] ±510.32
Group 4	2133.60 ±196.26	2754.00 ±224.39	3426.00[a] ±295.17
Group 5	2123.10 ±113.80	2747.00 ±157.90	3360.00[ab] ±145.27

[ab]Different literals in the same column indicate difference ($p<0.05$). [1]Treatments, Group 1 and Group 2: inoculated with *E. coli* and extracts of *P. auritum* and *O. basilicum*, respectively, Group 3: inoculated with *E. coli* without extracts, Group 4: inoculated with *E. coli* and Enrofloxacin at 10% y Group 5: negative control. [2]Values are represented as mean ±S.D.

With respect to relative weights of the organs (Table 5), there was a difference in liver weight ($p<0.05$). The highest relative weight of the liver was recorded in group 3 (2.58%) and the smallest in group 5 (1.61%). In groups 1 and 2 treated with *P. auritum* and *O. basilicum,* respectively, the liver relative weight was superior ($p>0.05$) to 2.0% and lower ($p<0.05$) than in group 3, which suggests a hepatomegaly caused by colibacillosis. *P. auritum* and *O. basilicum* had a beneficial therapeutic effect on chickens, decreasing the inflammatory response caused by pathogenic stimulus reflected in lower weight of the liver, with regard to group 3 (negative control). Several protein groups are synthesized in the liver as response to microbial challenge that can increase the relative size of this organ, which allows the estimation of the intensity of an inflammatory response (Korver *et al.,* 1998; Willcox & Bodeker, 2000; Chávez *et al.*, 2015).

Table 5 – Relative weight (%) of chicken organs inoculated with avian *E.* coli serotype O2, treated with extracts of leaves with phytobiotic properties.

	Treatments[2]				
	Group 1[1]	Group 2	Group 3	Group 4	Group 5
PC[3]	0.50 ±0.06	0.48 ±0.08	0.48 ±0.13	0.46 ±0.05	0.51 ±0.05
PH	2.06^{b} ± 0.32	2.01^{b} ±0.42	2.58^{c} ±0.81	1.87^{ab} ±0.32	1.61^{a} ±0.09
PB	0.07 ±0.03	0.07 ±0.03	0.09 ±0.03	0.06 ±0.01	0.06 ±0.00
PTGI	6.11^{ab} ±0.92	6.30^{b} ±0.61	6.45^{b} ±1.94	5.39^{a} ±0.86	6.94^{b} ±0.66
PBF	0.13^{ab} ±0.04	0.18^{c} ±0.05	0.10^{a} ±0.05	0.14^{bc} ±0.02	0.16^{bc} ±0.03
PP	0.69^{b} ±0.12	0.71^{b} ±0.13	0.73^{b} ±0.20	0.70^{b} ±0.15	0.53^{a} ±0.09
PR	0.69 ±0.20	0.66 ±0.14	0.72 ±0.30	0.61 ±0.22	0.76 ±0.10

[abc]Different literals in the same row indicate difference (p<0.05) [1]Values are given in percentage (%)= [(organ weight/body weight) X 100] and represent the mean ±S.D. [2]Treatments, Group1 and Group 2: inoculated with *E. coli* and extracts of *P. auritum* y *O. basilicum*, respectively, Group 3: inoculated with *E. coli* without extract, Group 4: inoculated with *E. coli* and Enrofloxacin at 10% and Group 5: negative control. [3]Organs, heart= PC, Liver= PH, Spleen= PB, Gastrointestinal track= PTGI, Bursa of Fabricious= PBF, Lungs= PP, kidneys= PR.

Differences (p<0.05) were found in the relative weight of the gastrointestinal track (WGIT). The greatest WGIT was recorded in group 5 (6.94%), while the lowest was in group 4 (5.39%); regarding groups 1 and 2, they had similar weights (p>0.05). The relative weight of bursa of Fabricious (WBF) was greater (p<0.05) in group 2 (0.18%), followed by group 5 (0.16%) and the lowest weight was in group 3 (0.10%). The relative weights of lung (WL) were only different (p<0.05) in group 5 (0.53%) with regards to the other groups, which means that inflammatory response in chickens inoculated with *E. coli* increased in WL, coinciding with Lau *et al.* (2010), who reported that chicken inoculated with *E. coli* O78:K80 showed higher weight (p<0.05) in lungs compared to those not inoculated. It is important to mention that the route of inoculation was intratracheal, favoring lesions originated in the respiratory system.

The quantification of CFU ($\log_{10}$) of *E. coli* isolated from organs (Table 6) was greater (p<0.05) in the lungs of group 4 with Enrofloxacin at 10%, bacterial growth can be translated as a possible resistance to the use of some antimicrobials and where in field *E. coli* has already been reported resistant to this product (Itza-Ortiz, 2018 personal communication). The lowest concentration of *E. coli* was observed in groups 2 and 1, regarding the other groups (p<0.05); the aforementioned supports phytobiotic activity of *P. auritum* and *O. basilicum* extracts on avian *E. coli* serotype O2.

It is possible that secondary metabolites present in *P. auritum* extracts or oils, such as safrole, besides synergism with other substances as thymol, carvacrol, myristicin, linalool, borneol, camphor, cineol, methyl *eugenol* and a wide variety of benzene components, are inhibiting bacterial growth (Domínguez *et al.,* 1962; Oliveira *et al.,* 2004). However, between phytobiotic extracts studied, the smallest growth of *E. coli* was obtained in *O. basilicum*, due to its active components, such as: β-linalool, estragole, linalool, 4-allyl anisole and eugenol; besides having phenolic acids derived from cinnamic acid and flavonoids (Acosta *et al.*, 2003; Roldán *et al.*, 2010), they form complexes with proteins and polysaccharides present in the external membrane of the cell, destabilizing the function of the membrane and cellular wall, causing microorganism death (Aguilar *et al.,* 2007). Lee *et al.* (2003) report that isoprene, chemical unity of terpenoids, which derive in three phenols, with phytobiotic properties: thymol, carvacrol and eugenol, can dissociate the external membranes of Gram negative bacteria, such as *E. coli* and *S. typhimurium*.

Table 6 – Concentration ($\log_{10}$ CFU g^{-1}) of avian *E. coli* serotype O2 in pool of heart, liver and spleen, and lungs of inoculated chicken and treated with extracts of leaves with phytobiotic properties.

	Treatments[2]				
	Group 1	Group 2	Group 3	Group 4	Group 5
Pool	4.06 ±0.86[1]	2.64 ±0.47	5.75 ±3.03	6.03 ±2.87	0.00 ±0.00
Lungs	3.92 ± 0.97^{ab}	1.87 ± 0.87^{a}	6.32 ± 1.48^{bc}	7.36 ± 1.59^{c}	0.00 ±0.00

[abc]Different literals in the same row indicate difference (p<0.05). [1]Values represent the mean ±S.D. [2]Treatments, Group1 and Group 2: inoculated with *E. coli* and extract of *P. auritum* and *O. basilicum*, respectively, Group 3: inoculated with *E. coli*, without extract, Group 4: inoculated with *E. coli* and Enrofloxacin at 10% and Group 5: negative control.

There was higher frequency of aerosaculitis (Table 7) in chickens inoculated with *E. coli*, because the route of inoculation was intratracheal. As shown, it was more severe in group 3. Aerosaculitis observed in group 3,

Table 7 – Morbidity of chickens inoculated with avian *E. coli* serotype O2, and treated with extracts of leaves with phytobiotic properties.

	Treatments[1]				
Sign	Group 1	Group 2	Group 3	Group 4	Group 5
Aerosaculitis[2]	8/10	7/10	10/10	8/10	0/10
Pericarditis	4/10	3/10	7/10	4/10	0/10
Perihepatitis	4/10	4/10	7/10	4/10	0/10

[1]Treatments, Group1 and Group 2: inoculated with *E. coli* and extract of *P. auritum* and *O. basilicum*, respectively, Group 3: inoculated with E. coli without extracts, Group 4: inoculated with *E. coli* and Enrofloxacin at 10% and Group 5: negative control. [2]Number of positive chickens with signs against number of chickens studied, is shown.

coincides with the reported by Lau *et al.* (2010), who indicate that 100% of inoculated chickens with *E. coli* serotype O78 showed this pathology. Presence of pericarditis and perihepatitis indicate a systemic infection and it was similar in groups 1 to 4; similar data were reported by Lau *et al.* (2010), in presence of pericarditis and perihepatitis in chicken inoculated with *E. coli* serotype O78:K80. Likewise, Rawiwet and Chansiripornchai (2009) report greater incidence of aerosaculitis than pericarditis and perihepatitis in chicken inoculated with *E. coli* serotype O78.

Mortality in group 3 was 20%, 10% for group 2 and for the other groups (1, 4 and 5) there was no mortality; mortality rate of field, caused by *E. coli* infection could be 10% (Shane, 1981; Wray *et al*., 1996).

Histological lesions were more severe in group 3 than in groups 1, 2 and 4. Lung parenchyma showed multiple granulomas with necrotic center located in the lumen, surrounded by heterophils, lymphocytes, macrophages and giant cells (Figure 1); the aforementioned was not observed in groups 1 and 4; being less frequent in group 2. Air capillaries were more thickened in group 1. Histological findings suggest a restorative process of the damaged tissue; mainly in group 3, where necrotic tissue is being replaced by granulation tissue. Also, there is an immunological response, due to the presence of giant cells, macrophages, lymphocytes and few heterophils (Woolcock, 1979; Ridell, 1987). Heterophils are cells whose main function is the phagocytosis of several particles such as bacteria and other microorganisms (Banks, 1996), the lowest amount observed of granulation, can be due to the fact that necropsy of chickens was performed nine days after inoculation and heterophil infiltration occurs in early infections, in the first 48 hours post inoculation (Barnes *et al.,* 2003).

Plant extracts with phytobiotic activity can act in the same manner as GPA, preventing immunological stress and its metabolic changes caused by the bacterial inoculum preventing immune activation (Roura *et al.,* 1992). It is important to mention that there were

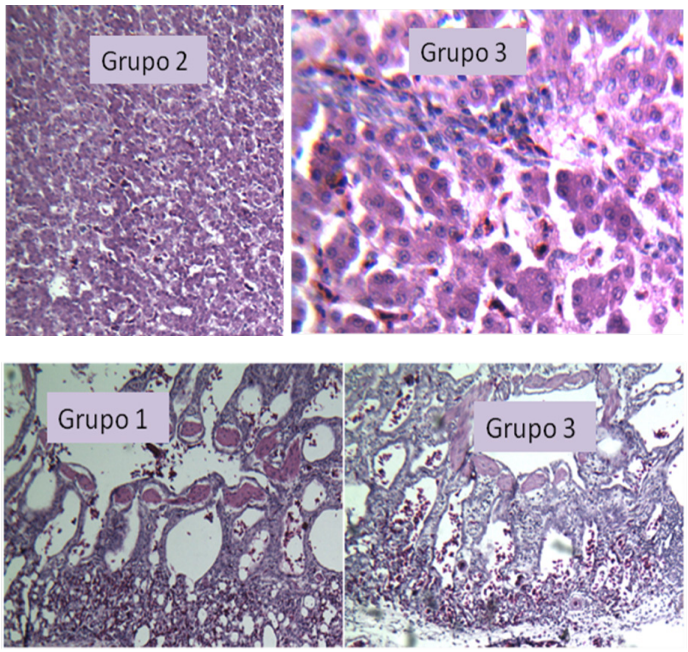

Figure 1 – Histological section of liver of Group 2 (chickens challenged with *E. coli* O2 and treated with extracts of *O. basilicum*) and Group 3 (chickens challenged with *E. coli* O2 and untreated).

lesions not associated with *E. coli*, but to a mixture of etiological agents; especially in the respiratory track, where an inflammation can respond to an unlimited number of infectious agents (Barnes *et al.,* 2003). The lesions observed in the liver of group 2 had the largest organization of hepatocyte cords in the hepatic parenchyma, possibly due to the therapeutic effect of *O. basilicum* extracts. Moderate multifocal hepatic lipidosis was observed in groups 1 and 2; group 3 showed mild multifocal. Hepatic lipidosis is caused by micotoxins in food, not by *E. coli* (Ridell, 1987); which means that chickens in group 3 had lower amount of food intake.

CONCLUSION

Extracts of the leaves of *P. auritum* and *O. basilicum* had phytobiotic activity on *E. coli;* with concentration higher than 8% and DIZ was observed above 10 mM. The solvent used in the extraction of active components of the plant can be an important factor in its activity,

since larger DIZ were obtained with the alcoholic extracts; in addition to this, the aqueous extract at 10% of *P. auritum* promoted food intake, reflecting a weight similar to commercial antimicrobial, which decreased the severity of the infection. Total *E. coli* count in lungs, heart, liver and spleen in chickens with *P. auritum* and *O. basilicum* extracts was lower than the obtained in chicken groups treated with commercial antimicrobial. Macroscopic lesions were observed in chickens with evidence of systemic illness, caused by bacterial inoculation; nevertheless, a greater number of repetitions are necessary to obtain the therapeutic effect caused by the extracts, since lesion classification according to its severity could be provided.

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