Effect of Cymbopogon Citratus (DC.) Stapf., Plectra Thus Amboinicus (Lour.), Tilia Cordata (Mill.), Lippia Alba (Mill.) and Ocimun Bacilicum (L.), To Control Escherichia Coli in Broiler Chickens

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Abstract

This study was conducted at both field and laboratory at the College of Agricultural Sciences at the Technical University of Machala (Ecuador). The objective was to demonstrate the effect of the infusions of Cymbopogon citratus (DC.) Stapf., Plectra thus amboinicus (Lour.), Tilia cordata (Mill.), in the drinking water of broiler chickens Cobb 500, as well as pure leaf extract of Plectranthus amboinicus (Lour.), Lippia alba (Mill.) and Ocimun bacilicum (L.), for the control of E. coli. In base of the results it can be concluded there is an effect on the inoculated E. coli when the infusions were offered to broiler chickens, and that the extracts used in this research were efficient at laboratory level to inhibit bacterial growth in a selective growth medium.

Keywords: Infusion, pure extract, colimetry, Mac Conkey Agar.

1. Introduction

With the modernization at the production farms, and the increase on the densities of animal population, a notable increase in reports of pathogens causing production losses, and occasionally, the development of "resistances" with their respective economic impact has been observed. This has been a major concern in grow out farms, due to stringent food safety measures, and proper application of withdrawal times for the drugs and chemicals used as promoters, preventive or curatives in the feeds and drinking water of farmed animals. Based on the above mentioned, in the poultry industry, from a considerable number of diseases, Colibacilos is stands out, causing economic losses worldwide, caused by *E. coli*, a gram negative bacillus that colonizes the intestines of animals within a few hours after birth (Rodríguez, 2002).

E. coli is generally a saprophytic inhabitant of the gut, and depending on their serotypes may be a causative agent of diarrhea in infants, adult animals, and man (Stanchi, 2007). The problem becomes complex with the pollution at the farms, and the appearance of bacterial resistance (Falcón et al, 2010; Joshi, 2012; Zeryehun et al., 2013; Cota et al., 2014) together with the ban on the use of antibiotics as growth promoters (Cepero, 2005) in the poultry industry in Europe and other countries, who decided not to use these substances due to the residues thereof in consumer products of animal origin, resulting in a positive change that other nations are considering to establish it as part of their food sovereignty, and thereby triggering studies of possible replacement alternatives (Camino et al., 2004; Ortiz y Pereira, 2012; Gonzáles et al., 2013; Chiriboga et al., 2015), which in most cases are promising and effective, highlighting among them the use of medicinal plants in animal production (Ayala et al., 2006; Gurgel et al., 2009; González et al., 2011; Lambrecht et al., 2013; Silva et al., 2014; Chiriboga et al., 2015).

The use of plant extracts, both from dry and fresh parts, essential oils, boiled, infusions among others, has shown to have an effect in controlling bacteria (Roldán, 2010; Bastos et al., 2011; Silva et al., 2014), therefore, for this study, easily spread medicinal plants with possible antibacterial potential were used. *Cymbopogon citratus* (DC.) Stapf, known as hierba Luisa in Ecuador is an herbaceous plant present in the 4 regions of the country, perennial, aromatic and robust that spreads by rhizomes epigean, tillering and becomes dense, belongs to the family *Poaceae* (*Gramineae*). The aromatic leaves, fragrant are elongated as strips 30 to 70 cm long from the base of the sheath, 1 to 2 cm wide, light green 7.5GY-4/4, rough with serrated margins tiny visible to the stereoscope. The flowers are grouped in spikes and bend like leaves. It is commonly known as hierba Luisa, hierba limón, toronjil de caña, limonaria, limoncillo, zacate de limón, té de limón, caña de limón, caña santa, lemongrass, hierba de la calentura, paja de limón, pasto limón, cedrón paraguayo, cedrón. In France it is called "citronnelle", and "verbena" anywhere else. It grows in tropical, subtropical, temperate and warm climates. Its development demands a high number of light hours (8 to 12). Resists strong winters in well-drained soil, it supports rains but no excess water. The intensity of the aroma of the leaves varies from month to month during the year, with the months of low rainfall, higher light intensity and high temperature, in which more aroma emerges from its leaves, while in wetter times of low temperature leaves are little aromatic. This particular feature of this plant indicates that environmental factors directly affect the expression of genes responsible for the production of active ingredients or essential oils (Quevedo Guerrero, 2016).

Plectranthus amboinicus (Lour.) is a perennial herb, robust, fleshy leaves, the adaxialface is green 5GY-4/4 and green 5GY-6/4 in the abaxial face, with abundant short hairs on the beam, very long and dense on the underside, very fragrant, the flavor is very similar to the common oregano. The width of the leaves is 4 to 7,5 cm and length of 3,6 to 6,8cm, petioles 1 to 2,5 cm long purple and green. This plant in Ecuador is known as Oreganón and it has many other common names in different countries (oregano francés, menta mexicana, oregano indio, oregano brujo, etc.). It is a herbaceous perennial plant, branchy, fragrant, with angular and fragile stems, belonging to the order *lamiales*, family *Lamiaceae*; originating in the tropical regions of East Asia and Southeast Africa. It shows a semi-erect like growth and can reach up to 1 m in height; flowers with dynamos stamens, inflected, filamentary sometimes united below, they are bilabials in violet color, and are grouped in whorls that form terminal spikes along 10 to 20 cm with bracts 3 to 4 mm in length and corolla in a pale lilac or pink and blue colors. This plant is grown since long ago in countries like India, France, Spain, Cuba, Mexico, Honduras, Ecuador, etc. and it has been attributed medicinal properties (Quevedo Guerrero, 2016).

Tilia cordata (Mill.), known as tilo, It belongs to the Family *Tiliacce*, Genus *Tilia* and species *T. cordata* Mill. Deciduous tree up to 30 m. tall, with a greyish bark trunk, little cracked lengthwise and wide, regular, subglobose or ovoid. Its leaves are characterized by alternate, deciduous, simple, petiolate, stipules large, deciduous, and 3-10 cm blade length, broadly ovate, cordate at base, abruptly acuminate, margin irregularly dentate-serrate, glabrous by beam with tufts of reddish and simple hairs in the axils of nerves on the underside. The flowers are hermaphrodite, actinomorphic, fragrant, 5-mere, gathered in pedunculated erect peaks, with an tongue-like oblong bract, obtuse, welded in less than half peduncle; dialisépalo calyx, yellowish dialipétala corolla, absent estaminodios, numerous stamens and a gynoecium pentacarpelar sincárpico súpero ovary (Árboles y arbustos, 2016). The chemical composition of Tilo is characterized by the presence of Polyphenols: flavonoids (1%), phenyl-Carboxylic Acids: caffeic, chlorogenic, p-coumaric, proantocianidoles, tannins, mucilages (3%), phenyl-Carboxylic Acids: caffeic and free derivatives and esterified gallictannins and catechists, heterósidos coumarin (Ferozo Site, 2014).

The infusion of linden flowers is used in the treatment of colds, febrile illnesses, rheumatic diseases and respiratory system (used primarily as a sedative for coughs and colds), it is also used for disorders of the nervous system, as a sedative and anxiolytic due to the presence of monoterpénicos oxygenated derivatives in the essential oil (Fresquet and Tronchoni, 2010).

Lippia alba (Mill.), known in Ecuador as mastrante is an aromatic and medicinal species native to America, used in folk medicine, it belongs to the family *Verbenaceae*, Genus *Lippia*, especie *L. alba* (Mill.) N.E.Br. ex Britton & P. Wilson., is a perennial shrub up to 2,50 m high, very branched with branches of determinate growth, puberulent and strigose, leaves are oblong, more pubescent beneath, membranous, conspicuously venous, lanceolate, green color 5GY3/4 in the adaxial face and olive color 5GY4/4 in the abaxial face, measure from 1,5 to 6,5 cm long and 0,7 to 3,1 cm wide, acute to obtuse apex, dentate margin, cuneate base, being opposite or sometimes alternate, petioles measure 10 to 14 mm long. Inflorescence axillary, capitated, pedunculated, hipocraterimorfa corolla, with a whitish tube with a yellow or white throat, hermaphrodite flowers, zigomorfas, with shades of white, pink or blue-purple, bilabiate cup, 1/3 respect to the length of the corolla, pubescent. Ovate bracteoles ovate, 3-6 mm long. Didívamos stamens, ovate anthers with parallel teak. ovary superior, biceldado, basal eggs, one egg per cell. The Nut is divided into two pyrene at maturity. The seeds have no endosperm (Quevedo Guerrero, 2016).

Ocimun bacilicum (L.), known as albahaca, is an annual shrubby plant that belongs to the family *Lamiaceae*, Genus *Ocimum*, comprising between 50 and 150 species of herbs and shrubs, which is distributed in tropical and subtropical regions of Ecuador and other countries in the region. It is a highly branched low growing plant (between 40 and 140 cm), with opposite glossy green leaves 7.5GY-4/4 on the beam and olive green 5GY-5/4 on the underside, lance-shaped leaves with acute base, jagged and silky texture, measuring 2 to 9 cm long and 1,5 to 5,5 cm wide. It emits terminal flower spikes with white tubular flowers four stamens and pistil resting on the lower lip of the corolla. After insect pollination, the corolla falls and four round achenes develop inside the lipped cup, which will be the seeds, the outer pericarp (or outer epidermis) seed when immersed in water, or during rain, soon swells forming a gelatinous mass that repels insects. Brasil in Ecuador and other Latin American countries has traditionally been used as a medicinal herb in the treatment of headaches, cough, diarrhea, intestinal parasite control and kidney diseases, it also has a long history as a culinary herb, thanks to the aromatic characteristics of its leaves, which add a distinctive flavor to many recipes of traditional meals. It is also considered an extraordinary source of essential oils and aromatic compounds containing biologically active constituents possessing nematicidal action, work well as insect repellents and shows antibacterial activity (Quevedo Guerrero, 2016).

The objective of this study was to demonstrate the effect of infusions of *C. citratus* (DC.) Stapf., *P. amboinicus* (Lour.), *T. cordata* (Mill.), in broiler chickens Cobb 500, and action pure leaf extract of *P. amboinicus* (Lour), *L. alba* (Mill.) y *O. bacilicum* (L.) in bacterial growth plates, for the control of *E. coli*.

2. Materials and Methods

This study was conducted at both field and laboratory at the College Agricultural Sciences at the Technical University of Machala. The results are the outcome of adding both pure extract and infusions of medicinal plants.
For the field research where infusions were applied, standard rules for animal husbandry were adopted, trying as much as possible to receive the chickens in a barn disinfected with formalin, iodine and quicklime. No antibiotic was given as preventive in the drinking water or the feed; however a basic vaccination program was applied (New Castle La Sota and Gumboro D78). The chickens were received on the wood shavings litter with newspaper on the surface for the first 3 days, and later they were left only with the litter. The study took 42 days, a total of 160 mixed Cobb 500 chickens were evaluated, there were 4 treatments with 4 replicates of 10 chickens each.

The first treatment (control or T1) not any infusion was given, while for treatment 2 (T2), treatment 3 (T3) and treatment 4 (T4) 3cc infusion (10%) of *T. cordata* Mill, *C. citratus* (DC.) and *P. amboinicus* (Lour.) per liter of drinking water was added, respectively. The field methodology consisted in collecting 3g stool from each treatment with their respective replications. At the laboratory a concentrate from each replication was done. Then 1g of each was taken and diluted and screened with 10 cc of sterile saline in a petri dish. From this 1cc was taken with a syringe and placed in a dilution 10x-2 until a 10x-9 dilution, and plated in sterile nutrient culture media (MacConkey Agar). Samples were taken at day 8, 22, 26 and the inoculation of the bacteria was on the same day a few hours later.

The plating after inoculation was on day 15, 36 and 42. For the inoculation, pathogenic colonies from a culture of *E. coli* were taken and diluted in 200 cc in a sterile culture media, from which 1cc was orally administered by means of a pipette to each chicken. To record the weight of the chickens a scale (CAMRY model EK9332-F302) with ± 1g accuracy was used.

In the laboratory experiment, where pure extracts (from leaves) of *P. amboinicus*, *L. alba* and *O. bacilicum* a simple extraction method was applied. For this 100 g of organic material was taken and crushed in a sterile mortar, then the liquid obtained was filtered in a beaker and then stored in a sterile plastic bottle, obtaining 88g, 69g and 26g of pure extract of Oreganón, Albahaca y Mastrante, respectively. The culture selective media for enterobacteria (MacConkey Agar, DIFCO), was prepared by diluting 50g of the media in 1000 cc of distilled water. Once the culture of *E. coli* was obtained, the same culture medium used for isolating the microorganisms were prepared, but to each one concentrated infusions of the three extracts were added at concentrations of 0,5%, 1% y 2%, then the prepared medium was plated and *E. coli* was added in each Petri dish, and finally they were left incubating at 38 °C, for 24 hours.

2.1. Statistical analysis

The applied statistical analysis was performed according to Sokal and Rohlf (1995), to determine whether statistically significant differences between treatments existed, analysis of one factor (ANOVA) was used, evaluating the effectiveness of the infusion. The program STATGRAPHICS Centurión XV.I. was used.
For laboratory results with extracts not any statistical analysis was carried out.

3. Results and Discussion

3.1. Effect of infusions

When comparing the live weight of chickens in each treatment by taking the average of every week, an arithmetic difference is found, but with no statistically significant effect (Table 1 and 2, Figure 1), similar data was found by Shiva et al. (2012), using the essential oil of oregano as a potential growth promoter in chickens. The result of the colimetry carried out on day 15 after inoculation of *E. coli* showed that higher CFU amount were present in T2 group (Tilo) while the lowest CFU was recorded with T4 when compared with T1; on day 22 and 26 prior to inoculation and 36 post-inoculation it was observed that the highest CFU is from T1. By day 42 after inoculation the trend for all of the treatments is somewhat similar (Table 1, Figure 2).

This could demonstrate there is an effect with the addition of plant infusions, similar to the antibacterial effect found by Naik et al. (2010) when using essential oil of *C. citratus*; Vatľák et al. (2014) with methanolic extracts of *T. cordata*; and Chiriboga et al. (2015) with an infusion of *P. amboinicus*. The observed mortality was not related somehow to the treatments. Some chickens were randomly selected for macroscopic abnormalities and sacrificed for necropsy. The T1 group (control) presented very marked hemorrhagic lesions and irritation in the intestinal mucosa, macroscopically different to what was observed in the groups T2 (Tilo), T3 (Hierbaluisa) and T4 (Oreganón), where the lesions were minimal. Betancourt (2012) and Shiva et al.(2012) mentioned in their remarks a greater length of villi and shallow Lieberkuhn crypts, associating this effect with the carvacrol and thymol content of oregano, which has bactericidal effects.

3.2. Effect of pure extracts

It was observed that the higher is the concentration the higher is the effect, as the bacteria reduced their growth significantly when compared to the control which was plated in media without extracts showing a countless bacterial growth covering the entire area of the Petri dish , thus being demonstrated the antibacterial effect *P. amboinicus* (both at 0,5% and 1%,and quite clear at 2%), *L. alba* had little effect at 0.5% and 1% in the control of bacterial growth, but at 2% showed a limited antibacterial effect; *O. bacilicum* had a similar effect to the *L. alba* at the different concentrations of the extract used. Something similar was demonstrated by Galvão et al. (2013) when using essential oils of species *Plectranthus*; Machado et al. (2014) with essential oils of *L. alba;* and Calderón and Torres (2014) with the aqueous extract *O. bacilicum*.

4. Conclusions

The addition of infusions of Hierba luisa (*C. citratus*), Oreganón (*P. amboinicus*) and Tilo (*T. cordata*) for the control of *E. coli* inoculated in broiler chickens showed an effect that should be deeper studied because the present results were obtained with small infusion volumes (3 cc) diluted in one liter of drinking water.

The pure extracts from fresh leaves of *P. amboinicus* (Oreganón), *L. alba* (Mastrante) and *O. bacilicum* (Albahaca) at the laboratory scale experiment carried out in this study showed a control in the population of *E. coli* by means of a bactericidal-bacteriostatic effect along with an increasing in its concentration (2%). These results could allow carrying out the same experiment to field application and thus testing the infusions at different concentrations to analyze their effects.

5. Recommendations

1. Determine which active ingredients are present in the infusion using as a standard the pure extracts and water.
2. In field studies replications should be performed for each treatment in colimetry to statistically confirm the results obtained in this study.
3. At laboratory level, more replicas are recommended for each pure extract in order to compare whether the effect on the control of *E. coli* is constant, furthermore a trial where the CFU could be quantified should be performed.
4. Demonstrate if the harvest time of the leaves of the medicinal plants studied in this research have a statistically effect on the content of their active ingredients.

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8. ANNEX

Table 1: Average live weights per weeks, with colimetry (in days) before and after inoculation of the bacterium *E. coli*, obtained with the addition of infusions of *C. citratus, P. amboinicus* and *T. cordata,* in the drinking water of broiler chickens Cobb 500.

AVERAGES OF WEIGHTS ALIVE AND COLIMETRY (CFU)								
Treat.[1]	**Body weight (g)**	**N.Sig.[4]**	**Day 8 (A.I.)[2]**	**Day 15 (P.I.)[3]**	**Day 22 (A.I.)[2]**	**Day 26 (A.I.)[2]**	**Day 36 (P.I.)[3]**	**Day 42 (P.I.)[3]**
1	1245,1 ± 77,7 [a]	ns	Uncountable	7 x $10^{1°}$	30,75 x $10^{1°}$	139,11 x 10^{6}	222 x 10^{6}	19,07 x 10^{6}
2	1204,7 ± 77,4 [a]	ns	Uncountable	24,5 x $10^{1°}$	22,5 x $10^{1°}$	50,33 x 10^{6}	15,42 x 10^{6}	16 x 10^{6}
3	1224,4 ± 77,4 [a]	ns	Uncountable	7,7 x $10^{1°}$	56,6 x $10^{1°}$	38,44 x 10^{6}	18,2 x 10^{6}	27 x 10^{6}
4	1148,7 ± 77,4 [a]	ns	Uncountable	3,2 x $10^{1°}$	26 x $10^{1°}$	30,33 x 10^{6}	22,71 x 10^{6}	38 x 10^{6}

[1] Treat.: treatments, 1 Control, 2 infusion of *T. cordata* 10%, 3 infusion of *Cymbopogon citratus* 10%, 4 infusion of *Plectranthus amboinicus* 10%. [2] (A.I.): Days on which the result was obtained colimetry hours before inoculating of E. coli in chickens. [3] (P.I.): Days on which the result of the subsequent colimetry was obtained inoculating of *E. coli* in chickens. [4] N. Sig. : Significance level; ns: not significant (p > 0,05); * (p < 0,05); ** (p < 0,01); *** (p < 0,001)

Table 2: Analysis of variance (ANOVA) for the average weight of broiler chickens Cobb 500 among treatments (per week)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	1,22E+06	3	406054	0,55	0,6488
Within groups	6,96E+08	942	739321		
Total (Corr.)	6,98E+08	945			

Table 3: Extract amount obtained by grinding fresh leaves of *P. amboinicus, L. alba, O. bacilicum,* and its effect on the bacterial growth in culturing plates for *E.coli* (compared to a plate control without any pure extract added).

EXTRACTION DATA AND OBSERVED BACTERIAL GROWTH					
Scientific name	**Pes. h. fr.[1] (g)**	**Extr. P.[2] (g)**	**Ef. Ob.[3] (0,5%)**	**Ef. Ob.[3] (1%)**	**Ef. Ob.[3] (2%)**
Plectranthus amboinicus	100	88	**	***	****
Lippia alba	100	25,58	p.e.	*	***
Ocimun bacilicum	100	69,23	p.e.	*	***

[1]Pes. h. fr.: Fresh weight of plant leaves. [2]Extr. P.: pure extract obtained from the leaves according to Pes. h. fr.. [3]Ef. Ob.: Effect of bacterial growth was observed on the plate include pure extract in growth medium (0,5%, 1% y, 2%), the valuation is based on the observation; p.e: little effect observed, *: Regular effect observed, **: good effect observed, ***: very good effect observed, ****: excellent observed effect (stops the growth plate).

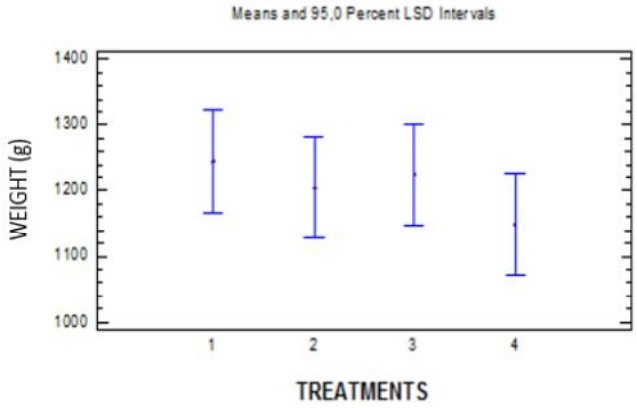

Figure 1: Average live weight of chickens Cobb 500 at six weeks in the different treatments (T1: control, T2: infusion of *T. cordata* 10%, T3: infusion of *C. citratus* 10%, T4: infusion of *P. amboinicus* 10%).

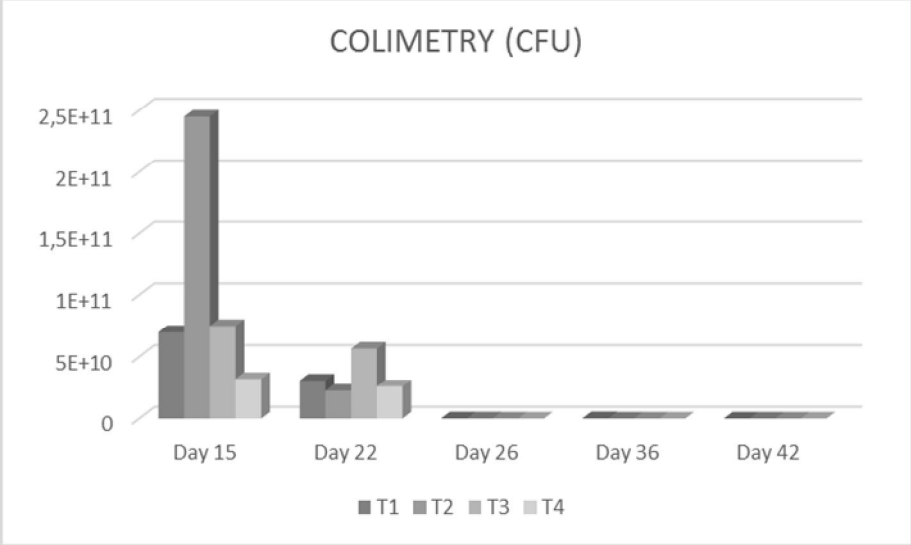

Figure 2: CFU in each treatment (different days), showing the effect of the extracts more clearly on day 15 and 22 with the tendency to get results similar to day 42 (when adding 3 cc of infusions at 10% of *C. citratus, P. amboinicus, T. cordata*.