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Effect of *Chrysactinia mexicana* Gray Extract on Laying Hens Organs Challenged with *Salmonella typhimurium*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JCGL, JMPR and GAF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BIJF, YJP, MACE, SLA and LOHA managed the experimental process and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Backyard poultry production systems are a very important source of meat and egg protein especially for children in rural areas.
Aim: Evaluate the biocide effect of *Chrysactinia mexicana* Gray extract on laying hens organs challenged with *Salmonella typhimurium.*
Study Design: *In vitro* and *in vivo* testing.

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Methodology: Two trials were conducted. In the first trial an *in vitro* experiment was performed with different solvents: aqueous, methylene chloride, ethanol and hexane for the plant extraction. Bactericide effect was determined. The second trial was an *in vivo* experiment 24 Plymouth Rock Barred hens, 21 weeks old (6 hens/treatment) were used with the following treatments: T1 control, no challenge and no plant extract; T2 control with challenge; T3 challenge and ethanol extract of *C. mexicana* and; T4 challenge and antibiotic. Colony Forming Units (CFU) of *S. typhimurium* in gizzard, ceca, crop and duodenum contents was measured.
Results: Bactericide assessment of *C. mexicana* with different solvent extracts resulted effective against *S. typhimurium* on concentrations between 20 and 25 mg/ml of extract. Ethanol extract has higher bactericide activity. Feed intake, total weight gain and final body weight was higher for T1 among the other treatments. Treatment 2 had the lowest trait performance. T3 and T4 had similar feed intake, total weight gain and final body weight response. The control group had lower CFU for crop, gizzard, ceca and duodenum compared with the other treatments. The highest content of CFU for all four organs was for the T2. The treatment challenged with *S. typhimurium* and *C. mexicana* extract had lower CFU for the organs than T2. Treatment challenged and with antibiotic showed better CFU counts than T3.
Conclusion: *C. mexicana* extract had a beneficial effect both *in vitro* and *in vivo* trials.

Keywords: Hens; Chrysactinia mexicana; Salmonella typhimurium.

1. INTRODUCTION

Common welfare of rural families in developing countries depends to a great extent on the benefits of backyard poultry production; it represents an important source of protein through meat and eggs, especially for children [1,2]. This kind of poultry production is severely affected by the incidence of enteric pathogens such as *Salmonella typhimurium*, which in turn leads to a high morbidity and mortality [3]. In Mexico, small-scale poultry production has shown high chick mortality (75%) and low egg production (78 per hen/year) [1,2]. *Chrysactinia mexicana* Gray, commonly known as false Damiane is a small shrub distributed throughout the southwest United States and central and northern Mexico [4]. Fig. 1 shows the major components of *C. Mexicana:* eucalyptol (41.3%), piperitone (37.7%) and linalyl acetate (9.1%) [5-8]. 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 [8]. Some researchers have been studying the *in vitro* antimicrobial effects of *C. mexicana*, which have demonstrated some bactericidal activity [9]. Other researchers evaluated the effect of *C. mexicana* in maize weevil (*Sitophilus zeamais* Motsch) and found that the leaf powder totally prevented F1 progeny from emerging [10]. In another experiment it was found that the essential oil of leaves of *C. mexicana* completely inhibited *Aspergillus flavus* growth [5]. Moreover, other researchers found that *C. mexicana* showed the greatest antimicrobial activity against the drug resistant strain of *Mycobacterium tuberculosis* [11]. Additionally, in México this plant is used to treat some enteric disorders in humans in the rural areas [9], however no reports are available regarding its use in poultry or other domestic animals. Because of the high cost of antibiotics and the low income of these kinds of producers, it makes the treatment of the broilers and hens a real problem. While alternatives are under investigation elsewhere, the objective of the present study was to evaluate the bactericide effect of *Chrysactinia mexicana* Gray extract *in vitro* and *in vivo* in laying hens challenged with *S. typhimurium*.

2. MATERIALS AND METHODS

The procedures for bird care were approved by the Ethics Committee on the use of animals of the UASLP (CONBIOETICA24CEI00820131212). Plant samples were chosen at blooming stage and without parasites. Samples were collected early in the morning to avoid the loss substances due to sunshine. The sampling method was using quadrats of 20 m^2. Simple random sampling was used for the hens in the experiment; hens were same age, weight and were not vaccinated.

2.1 *In vitro* Experiment

Plant collection was made from Guadalcazar village, located in a semi-desert area in the center zone of México. 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 different polar solvents (hexane, methylene chloride, and ethanol) and water. A complete randomized design was used for the following treatments: T1= ethanol; T2= aqueous; T3= Methylene Chloride; and T4= Hexane. Two hundred g of leaf ground powder samples were placed in a column by gravity or percolation and the solvent was added and sat for 48 h, using about 5 L of each solvent, then the samples were dried in an extraction chamber. The obtained extract was then concentrated at reduced pressure to 29℃ with a rotavapor (R-210/R-215 Buchi) [12,13]. Finally, the extract was dried by freeze drying process (cryodesicattion). To test the plant extracts a standard suspension of *S. typhimurium* (ATCC 14028) was prepared to meet the 0.5 Mc Farland standard equivalents to 10^8 CFU/ml concentration. To determine minimum inhibitory concentration (MIC) all solvent extracts were tested on agar diffusion assay [14]. These activities were established by the inhibition of the development of the bacteria (*S. typhimurium)* in Muller Hinton broth (MHB), and confirmed in Mueller Hinton Agar (MHA). Before running the assay, a maximum quantity of solvent was calculated in order to avoid interference in culture developing and it was set at 75 µL. Tubes containing 4.0 mL de MHB with the plant extract to be evaluated was added with 1 ml of the standard inoculum, and incubated at 35±2℃ for 24 h. For the minimum bactericide concentration (MBC), agar diffusion assay was used to test the bactericide activity for all solvent extracts, briefly; aseptically, bacteria (inoculation of standard microorganisms 10^8/ml) was swabbed onto the appropriate Petri dishes using a sterile cotton swab into Mueller Hinton Agar. Plant extracts were applied (75 µL) to a well that was cut into the agar in each Petri dish. Then the plates were set aside to allow diffusion in the media and were incubated at 35±2℃ for 24 h. Determination of bactericide activity was measured by the zone of inhibition in mm of bacterial growth at 24 h [9,15-17].

Table 1. Basal diet composition[1]

Ingredient	g/kg diet
Yellow corn 8%	572.32
Soybean meal 46.5%	304.79
Calcium carbonate 38%	99.50
Dicalcium phosphate	12.00
Mineral premix[2]	2.50
Vitamin premix[2]	2.50
Sodium chloride	2.50
Sodium bicarbonate	2.00
DL-Methionine 99%	1.64
Bacitracin methylene disalicylate	0.25
Chemical composition	
Metabolisable Energy, MJ/kg	11.98
Crude Protein, % (m/m)	17.98
Crude Fibre, %(m/m)	3.05
Ash, % (m/m)	13.56
Fat, % (m/m)	4.37
Methionine, % (m/m)	0.44
Lysine, % (m/m)	0.94
Threonine, % (m/m)	0.58
Phosphorus, % (m/m)	0.50
Calcium, % (m/m)	4.19

[1]Diet was offered ad libitum for the duration of the trial, and was formulated to meet or exceed all requirements for layer hens [18]; [2]Vitamin mix contained (per kg final diet) 0.8 mg thiamin, 2.2 mg riboflavin, 10.0 mg pantothenic acid, 11 mg niacin, 3 mg pyridoxine, 0.25 mg folic acid, 0.1 mg biotin, 0.004 mg vitamin B-12, 1500 IU retinyl palmitate, 300 ICU cholecalciferol, 5.0 IU all-α-tocopheryl acetate and 0.5 mg menaquinone. Mineral mix contained (per kg final diet) 0.1 mg selenium, 4 mg copper, 35 mg zinc, 30 mg manganese, 60 mg iron, and 0.35 mg iodine

2.2 *In vivo* Experiment

A complete randomized design was used. Twenty-four (24) Plymouth Rock Barred laying hens of twenty-one (21) weeks old were allocated in individual cages, 6 hens per treatment: T1: control, T2: control + *S. typhimurium* challenge, T3: control + *S. typhimurium* + *C. mexicana* extract and T4: control + *S. typhimurium* + antibiotic. The *C. mexicana* extract was administered orally via an esophageal cannula during 15 days at 20 mg/ml, *S. typhimurium* challenge was given same via at day one and five of the experiment. Feed and water was offered *ad libitum*, hens were not vaccinated. Feed was formulated to meet or exceed the National Research Council [18] requirements for layer hens (Table 1). Measured variables were: initial body weight, weight gain, final weight, feed intake and quantification of colony forming units per ml (CFU/ml) in gizzard, duodenum, ceca, and crop of hens which were slaughtered 21 d after *S. typhimurium challenge*. Briefly, the hens' carcasses were stored in plastic bags. From the different organs and using sterile scissors a small (approximately 1 cm) hole was cut, two milliliters of sterile PBS were pipetted into each organ. Only 2 to 4 mL of liquid was recovered. One milliliter was used for a culture. The colony counting procedure used was the membrane filter technique [19].

2.3 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 [20] software program. Bacterial numbers were converted to log CFU for statistical analysis.

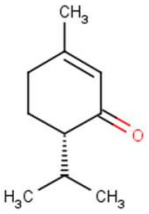
3. RESULTS

The extraction yields with different solvents were: aqueous 11%, ethanol 7.4%, methylene chloride 3.8% and hexane 3.5%. Bactericide assessment of *C. mexicana* with different solvent extracts resulted in an effective inhibition against *S. typhimurium* in concentrations between 20 and 25 mg/ml of extract. Fig. 1 shows that ethanolic extract has higher bactericide activity; on a 20 mg/ml concentration shows a higher ($P<0.05$) inhibition hale (17 mm) than the extracts with water, methylene and hexane, which showed similar inhibition hales but with a higher concentration (25 mg/ml). For the *in vivo* trial the ethanol extract of *C. mexicana* was chosen because it showed the highest *in vitro* bactericide effect. Feed intake, total weight gain, final body weight and feed conversion rate was higher ($P<0.05$) for T1 among the other treatments (Table 2). Treatment 2 had the lowest trait performance. T3 and T4 had similar ($P>0.05$) feed intake, total weight gain, final body weight and feed conversion rate response. The control group had lower ($P<0.05$) CFU for crop, gizzard, ceca and duodenum compared with the other treatments (Table 2). The highest ($P<0.05$) content of CFU for all four organs were for the T2. The treatment challenged with *S. typhimurium* and *C. mexicana* extract had lower ($P<0.05$) CFU for the organs than T2. Treatment challenged with antibiotic showed lower CFU counts than treatment challenged with *S. typhimurium* and *C. mexicana* extract.

4. DISCUSSION

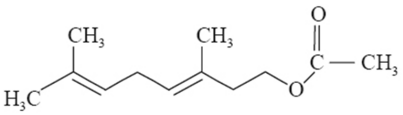
According to the national committee for clinical laboratory standards [12] an inhibition diameter of 15 mm or higher indicates susceptibility of the microorganism under evaluation. Since the used solvents were of different polarity, it can be said that the bactericide effect of the plant is due to different compounds present in the plant. In an experiment researchers [11] tested three different extract of *C. mexicana* aqueous, methanol and diethyl ether and found bactericide effect with two strains of *Mycobacterium tuberculosis*. Moreover, other researchers [21] reported that *C. mexicana* to have antimycobacterial activity. In another experiment [22] it was performed a phytochemical analysis and monoterpenes were identified. Also, an experiment with essential oil from leaves showed some antifungal activity [5]. The pharmacological effect of *C. mexicana* could be related to intracellular concentration regulation of Ca^{++} [23]. The action mechanism of majority *C. mexicana* components has been reported that eucalyptol inhibited contractions induced by carbacol [24, 25]. It also has anti-diarrheic activity [26]. Eeucalyptol 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 [27]. Finally, it has been reported that the essential oil from *Cymbopogon proximus* contains pipertitone as the highest compound (73.8%), this compound antagonizes the actions of serotonin and histamine, by interaction of its receptors [28]. According to the

results of this experiment the use of *C. mexicana* extract could be a good alternative, especially for low-income families in rural areas that cannot afford to purchase antibiotics for their hens.



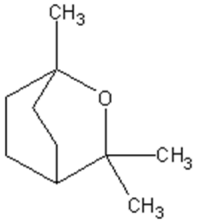
Piperitone $C_{10}H_{16}O$

6-Isopropyl-3-methyl-2-cyclohexen-1-one



Lynalyl acetate $CH_3CO_2C(CH=CH_2)(CH_3)CH_2CH_2CH=C(CH_3)_2$

3,7-Dimethyl-1,6-octadien-3-yl acetate



Eucaliptol C10H18O

1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane, 1,8-Cineole, 1,8-Epoxy-p-menthane

Fig. 1. Chemical structure of main active compounds of *C. mexicana*

Table 2. Means of hens performance and colony forming units [log CFU/ml] on crop, gizzard, duoden and ceca with different treatments

Treatment	T1	T2	T3	T4	SEM
Performance					
Initial body weight [g]	2403.7	2405.6	2404.7	2404.0	0.598
Final body weight [g]	2646.4[a]	2555.0[c]	2608.8[b]	2627.6[b]	4.152
Total weight gain [g]	242.7[a]	149.4[d]	202.1[c]	223.6[b]	0.581
Average daily gain [g]	11.5[a]	7.1[c]	9.6[b]	10.6[b]	0.012
Feed intake per day [g]	133.0a	113.3c	126.5b	128.2b	0.272
Feed conversion rate	1.91a	1.74c	1.81b	1.86b	0.013
Colony forming units [log CFU/ml]					
Crop	2.12 [d]	3.74[a]	3.30[b]	3.00[c]	0.034
Gizzard	1.20 [d]	2.92[a]	2.22[b]	1.70[c]	0.029
Ceca	1.68 [d]	3.03[a]	2.78[b]	2.28[c]	0.016
Duoden	1.50 [d]	2.86[a]	2.37[b]	2.14[c]	0.009

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

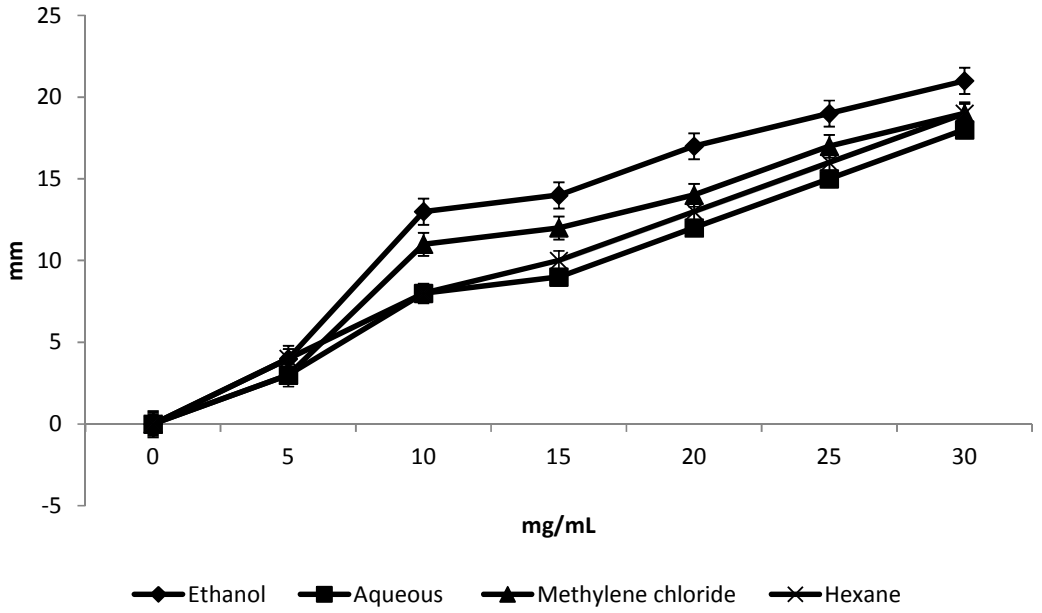


Fig. 2. *In vitro* means of zones of inhibition (mm) of *C. mexicana* with different extracts against *S. typhimurium*

5. CONCLUSION

In conclusion the *Chrysactinia mexicana* extract showed different effects according to the different solvents. In the *in vitro* trial the ethanolic extract was the extract that showed the highest activity against *S. typhimurium.* In the *in vivo* trail the performance traits showed similar results using *C. mexicana* extract and the antibiotic treatment.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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