WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 7, Issue 9, 17-29 Research Article ISSN 2278 – 4357

PLANT POLYEXTRACTS AGAINST AVIBACTERIUM PARAGALLINARUM AND PASTEURELLA MULTOCIDA, CAUSUAL AGENTS OF RESPIRATORY SYNDROME IN CHICKENS

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Article Received on
22 June 2018,
Revised on 12 July 2018,
Accepted on 03 August 2018
DOI: 10.20959/wjpps20189-12115

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ABSTRACT

The present investigation evaluates the capacity for *in vitro* growth inhibition of the casual agents of chronic respiratory syndrome among chickens, the bacteria *Avibacterium paragallinarum* and *Pasteurella multocida*, for the ethanolic plant extracts of the following plants: cinnamon (*Cinnamomum verum*), oregano (*Origanum vulgare*), redbird flower/ítamo/zapatilla del diablo (*Euphorbia [Pedilanthus] tithymaloides*), bougainville (*Bougainvillea glabra* Choisy), sierrecilla (*Mimosa lacerata*) and eucalyptus/Tasmanian bluegum (*Eucalyptus globulus*). From the ethanolic extracts, the phytochemical composition of the samples was determined, using chromogenic agents. Found were tannins, flavonoids, triterpenes, phenolic compounds and alkaloids. The *in vitro* anti-bacterial activity was assessed through the Kirby-Bauer technique. The sierrecilla extract showed the larger halo of inhibition with both bacteria, 2.16 cm for *Pasteurella multocida* and 2.43 cm for *Avibacterium paragallinarum*, using a concentration of 0.25 mg mL^{-1}.

KEYWORDS: Plant Polyextracts, Susceptibility, Bacteria, Chickens.

INTRODUCTION

In underdeveloped countries, such as Mexico, backyard production is an important system for the rural communities through which they obtain animal protein. It is an activity supportive of the family economy, carried out principally by housewives, children and elders. In the state of Guerrero, more than 75% of the rural families undertake this production(Juárez-Caratachea & Ortiz, 2001; Medina Cardena, Rejon Avila, & Valencia Heredia, 2012).

Respiratory illnesses represent one of the major problems in poultry production, owning to the high impact of infection agents and the environment conditions that augment the susceptibility of chickens(Mehmood et al., 2016). Amongst these diseases is avian cholera which infects both domestic and wild birds. It is caused by *Pasteurella multocida*, a Gram-negative bacteria that is nonmotile, non-spore-forming, pleomorphic ranging from 0.2 to 2 µm(Arce et al., 2012; Huber et al., 2015). When this disease presents itself in acute form, it is characterized by nasal secretion, facial edema, blackening of the comb and wattles, fever, and swelling (inflammation) of the head(Hafez, 2011; Mohamed, Mohamed, Ahmed, Ibrahim, & Ahmed, 2012; Shah, Kamboh, Rajput, & Korejo, 2008). Infectious rhinitis (coryza) is the second most frequent disease of bacterial origin, occurs principally in hens. It characteristically produces nasal discharge, sneezing, and facial inflammation(Arce et al., 2012; Islam et al., 2011; Muhammad & Sreedevi, 2015). It is caused by the etiological agent Avibacterium *paragallinarum*, a Gram-negative, non-spore-forming, nonmotile, coccobacillus bacteria ranging 1 to 3 µm in length by 0.4 to 0.8 µm in width(Hafez, 2011; Soriano Vargas & Terzolo, 2004).

For the prevention and treatment of respiratory illnesses in industrial poultry farming, there is heavy use of antibiotics, resulting in the appearance of bacterial resistance and the accumulation of some residuals of those medications in the flesh and eggs(Grande, Falcón, & Gándara, 2000; Sánchez, Muñoz, & Gutiérrez, 2012). For this problem, the World Organization of Health (WHO) has advocated for the search for natural alternatives, so to diminish the use of antibiotics, e.g., through promoting and developing alternative agents such as probiotics, prebiotics, organic acids and plant extracts(Lópes, Afanador, & Ariza, 2008). Medicinal plants has traditionally been used by all native cultures. Currently rural communities utilize them to cure illnesses in animals and humans, as is the case for some communities in the state of Guerrero where fowl are grown in a traditional manner. That is,

they are fed with maize, insects and some plants found in the countryside. These communities lack sanitary control. Respiratory illnesses occur with higher frequency(Gutiérrez-Triay et al., 2007). The importance of the use of plant extracts is for the chemical composition that give rise to different secondary metabolites, whose pharmacological properties are varied. They may act as antioxidants, antivirals, antimicrobials, antimutagenics, antifungals, antiparasitics, insecticides among other actions(Baños & Guillamón, 2014; Croteau, Kutchan, & Lewis, 2000; Huber et al., 2015; Villarreal et al., 2017). Some investigations note that in animals, plant extracts may act as growth promotors or preventive therapeutic agents since they augment the production of digestive enzymes and improve liver functions(Carro Travieso, Saro, Mateos, Díaz, & Ranilla, 2014; El Tawab, El-Hofy, Belih, & El Shemy; Naranjo & González, 2015). Given the discussion above, the object of this research was to evaluate the inhibitory activity on *Avibacterium paragallinarum* and *Pastuerella multocida*, bacteria associated with respiratory diseases in birds, by the plant extracts of the following plants: *Cinnamomum verumcanela* (cinnamon), *Origanum vulgare* (oregano), *Euphorbia [Pedilanthus] tithymaloides* (redbird flower/ítamo/zapatilla del diablo) *Bougainvillea glabra* Choisy (bougainville), *Mimosa lacerata* (sierrecilla) and *Eucalyptus globulus* (eucalyptus/Tasmanian bluegum).

MATERIALS AND METHODS

Sample collections of *Cinnamomum verumcanela* (cinnamon), *Pedilanthus tithymaloides* (redbird flower/ítamo/zapatilla del diablo), *Bougainvillea glabra* Choisy (bougainville), *Mimosa lacerata* (sierrecilla) and *Eucalyptus globulus* (eucalyptus/Tasmanian bluegum) was done in April, 2016 within the community Acahuizotla in the muncipality of Chilpancingo de los Bravo, Guerrero, Mexico (altitude: 820 meters; longitude: 17º 21' 38" and latitude: 99º 28' 02"). The climate is warm semi-humid with an annual average temperature of 22°C and an annual average precipitation of 1,373 mm(Ceballos *et al.*, 2010). The *Origanum vulgare* (oregano) was bought in the central market of the capital city Chilpancingo.

Table 1: Material used.

Plants analized	**Part of plant**
Bougainvillea glabra Choisy (bougainville)	Flowers
Cinnamomum verumcanela	Tree bark
Mimosa lacerata	Tree bark
Origanum vulgare	Leaves
Eucalyptus globulus	leaves
Pedilanthus tithymaloides	leaves

Plant Extract Acquisition

The samples were washed and dried in a TECNO DALVO convection oven at a temperature of 40°C for 3 days. They were subsequently placed in a Retsch Grindomix 200 electric mill to obtain pulverized samples. Immediately afterwards 300 grams of each sample was placed in 1000 mL amber glass bottles with 700 mL of alcohol. The bottles are left to settle. Each 72 hours a wash with alcohol was done. This procedure was repeated three times. Afterwards the samples were filtered, thus obtaining the alcohol extracts. Each sample's dried extract was obtained through a rotary evaporator.

For the phytochemical profile, 0.5 grams of dry material was placed in 12x100 mm test tubes with 20 mL of ethanol which subsequently were placed in an ultrasound bath for 15 minutes. Following that process, they were filtered. The phytochemical test were done utilizing chromogenic agents (substances that react, forming precipitates, foam, color changes, among other effects) described by(Galindo, Rosales, Murgueitio, & Larrahondo, 1989). For the assessment of the finished tests, a qualitative system of crosses was used to specify the presence or absence of groups of metabolites along the following criteria: high content or substantial presence (+++), notable presence (++), slight presence (+) and absence (-) (Galindo et al., 1989; D. García, Ojeda, & Montejo, 2003).

Bacterial Strains: The strains used in the biotests are *Avibacterium paragallinarum* and *Pasteurella multocida*, donated by the microbiological laboratory of the National Center of Animal Health Diagnostic Services (Centro Nacional de Servicios de Diagnóstico en Salud Animal), located in Tecámac in the state of Mexico.

The antibacterial activity of the extracts was evaluated with the Kirby-Bauer agar diffusion method(Bernal & Guzmán, 1984). The bacterial inoculations were prepared in 10mL Muller-Hinton (Bioxon®) culture medium. The culture plates were incubated at 37 °C for 24 hours. The inoculations were regulated with sterile saline solution until a turbidity of McFarland number 5 standards (1.5×10^8 UFC mL^{-1}) was obtained. The extracts were used at a concentration of 0.25 mg g mL^{-1}. The bacterial inoculations were spread upon the surface of the Muller-Hinton agar plates. Subsequently 6 mm diameter filter papers disks (Whatman Numerus 5), separately impregnated with 20 µL of each one of the prepared extracts and were placed on the surface of agar plates. For the positive control, a disc with 10 µg of ampicillin, a broad spectrum antibacterial pharmaceutical, was used. The plates were incubated at 37º C for 24 hours. Throughout this time, diameters of the zone of inhibition

were measured in centimeters. All the tests were done in triplicate. A comparison of averages with the Tukey's test with an α of 0.5 was done using the SAS version 9.1 statistics package.

RESULTS AND DISCUSSION

Phytochemical Analysis: The chemical characterization of the extracts showed slight differences in the composition of secondary metabolites of ítamo (*Euphorbia [Pedilanthus] tithymaloides*) and eucalyptus (*Eucalyptus globulus*) leaves. In bougainville (*Bougainvillea glabra* Choisy) flowers and in the bark of cinnamon (*Cinnamomum verum*) and sierrecilla (*Mimosa lacerata*), the presence of tannins, flavonoids, and triterpenes were observed in all the samples. Alkaloids were not found in the extracts of oregano (*Origanum vulgare*) and cinnamon (*Cinnamomum verumcanela*) (Table 1).

In the ítamo extract, tannins, flavonoids, triperpenes were detected, results similar to those of(Ortiz Sánchez, López González, Padró Rodríguez, & Velásquez Almenares, 2009) using fresh leaves. The bougainville extract presented metabolites similar to those reported by(Edwin, Sheeja, Toppo, Tiwari, & Dutt, 2007) for the leaves. The metabolites identified in the eucalyptus are similar to ones reported by(H. García, Quert, Becker, & Castiñeira, 2004; Gilles, Zhao, An, & Agboola, 2010). The cinnamon results were similar to (Herrera Arias & García-Rico, 2006).

The metabolite differences among species is due to the secondary metabolites being distributed heterogeneously in distinct parts of the plants (A. Á. García & Carril, 2011) Moreover, synthesis occurs in different parts of the cell. Some alkaloids and terpenes are synthesized in plastids; sterols, sesquiterpenes, and dolichols, in the endoplasmic reticulum; while the biosynthesis of some amines and alkaloids take place in the mitochondria (A. Á. García & Carril, 2011; Huber et al., 2015). Concentration of metabolites in the organs or tissues are affected by different factors, such as plant genotype, environmental factors, growth rate, soil nutrition, diseases among others (Ganjewala, Sam, & Khan, 2009; Varón & Granados, 2012).

Antibacterial Activity: In the evaluation of the antibacterial activity, extracts of sierrecilla, eucalyptus, oregano, bougainvillea, cinnamon and ítamo inhibited the growth of *Pasteurella multocida.* The extract of ítamo left a smaller halo compared to the positive control, 0.96 cm versus 1.6 cm. The extracts of sierrecilla and eucalyptus produce larger halos than the control (Figure 1).

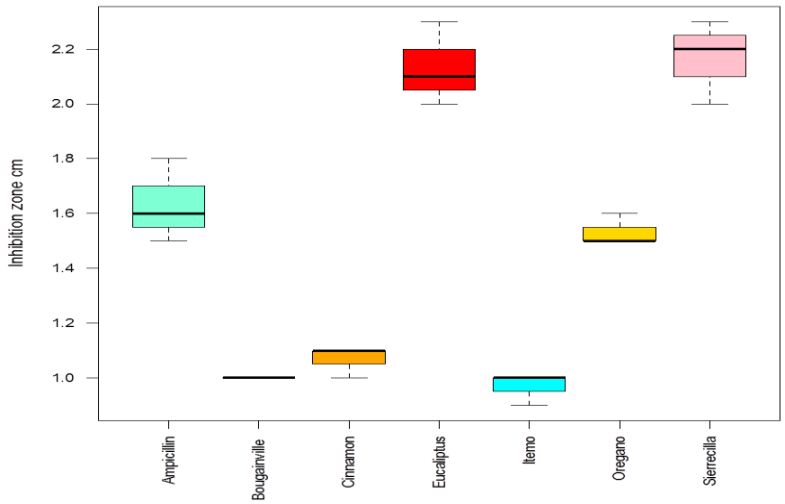

Figure 1: Inhibition zone in Pasteurella Multocida Induced by Different Plant Extract.

Due to the variety of the secondary metabolites presented in the secondary extracts of sierrecilla, eucalyptus, oregano, cinnamon, and ítemo, inhibition halos for *Pasteurella multocida* are present (as may be expected). Note that the extracts of eucalyptus and sierrecilla result in larger halos than ampicillin. (Carrillo, Chinchilla, González, Toledo, & Zambrana, 1997) encountered similar results with the extracts of *Coutaria hexandra, Petiveria alliceea, Cestrum lanatum and Jatropha curcas* with diameters of 9.5, 9.5, 7.5 and 8.75 millimeters respectively.

For the bacteria *Avibacterium paragallinarum*, the larger diameters of inhibition were those of oregano and sierrecilla. The positive control had an inhibition halo equal with the oregano extract (Figure 2).

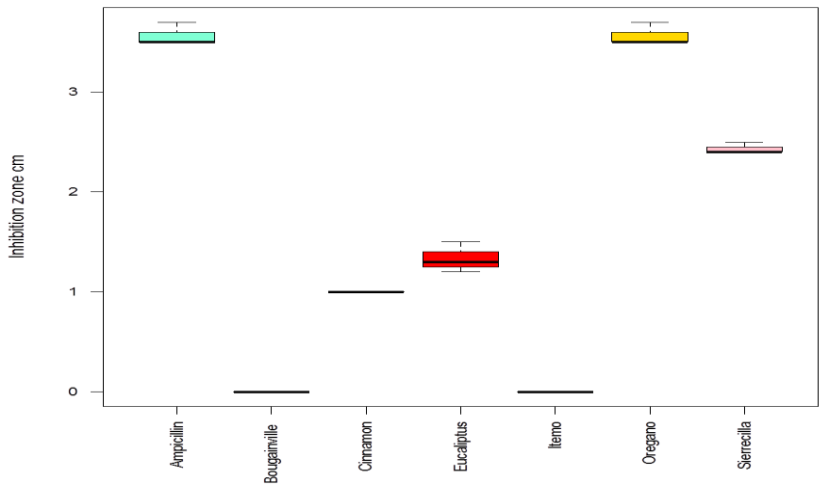

Figure 2: Inhibition zone in Avibacterium paragallinarum induced by different plant extract.

There have been no extract research into the growth inhibition of *Avibacterium paragallinarum*. Nevertheless, the antibacterial capacity of oregano and eucalyptus is attributed principally to thymol and carvacrol, both of which modify the physical structure of the bacterial cells, leading to the destabilizing of the cell membrane, changing its permeability and denaturalizing essential enzymes (Sesterhenn et al., 2015). In the investigation done by (Sebei, Sakouhi, Herchi, Khouja, & Boukhchina, 2015), the eucalyptus essential oil inhibit Gram-negative bacteria, halo diameters of 10-29 mm being obtained.

It has been reported for cinnamon extract that it inhibits the growth of Gram-positive bacteria better (Burt, 2004; Pastrana-Puche, Durango-Villadiego, & Acevedo-Correa, 2017), with respect to ítamo, reports indicate that Gram-negative bacteria have been reported to be less sensitive to ethanolic extract of fresh leaves, unless higher doses are used (Márquez-Vizcaíno, Mercado-Pérez, & Catalino, 2005), thus giving results similar to those obtained in this study.

The plant extracts of oregano, eucalytus and sierrecilla inhibited *Avibacterium paragallinarum* and *Pasteurella multocida*; this effect is seen since flavonoids were detected in the phytochemical screening. This antimicrobial activity may be due to flavonoids' ability to form proteinaceous interactions with intracellular proteins and interactions for formation of complexes with bacterial cell walls that involve cellular lysis (Pava, Sanabria, & Leal, 2017; Villarreal et al., 2017). Also observed were phenolic compounds. Their antibacterial activity is related to enzymatic inhibition by oxidized compounds, possibly through reactions of sulfhydryl groups or by the non-specific interaction of proteins (Domingo & López-Brea, 2003). Another common metabolite in the three species are tannins. Their capacity to inhibit the growth of bacteria is attributed to their ability to inactivate microbial adhesins, enzyme transport, and cellular envelope proteins (Perumal Samy & Gopalakrishnakone, 2010).

The Gram-negative bacteria are less sensitive to the plant extracts, given their bacterial complexity. To inhibit them, it is necessary to utilize higher doses than those used for Gram-positive bacteria (Fisher & Phillips, 2006; Wang et al., 2012). This is due to their antagonistic compounds which function as positive displacement-pumps of diverse substances. It also should be mentioned that porins impede the passage of secondary metabolites(Cruz-Carrillo, Rodríguez, & Rodríguez, 2010; Domingo & López-Brea, 2003; Fisher & Phillips, 2006; Mila-Arango et al., 2014; Wang et al., 2012).

Moreover, the quantity of secondary metabolites present in the extracts is very variable. Consequently, the activity may not be attributed to one specifically but to the combination of them over distinct parts of the microbial cell (Reyes-Jurado, Palou, & López-Malo, 2014).

Investigations into extracts and essential oils mention that the capacity to inhibit bacteria depends principally on three characteristics: the hydrophilic or hydrophobic character of the substance, the components present and the type of microorganism being attacked(Fisher & Phillips, 2008; Solórzano-Santos & Miranda-Novales, 2012).

The results of this investigation are preliminary data which must be confirmed by more sensitive techniques and furthered to evaluate the Minimal Inhibitory Concentration of each extract. It is of upmost importance to identify the metabolites of each plant and to prove the mechanisms of actions that are realized on the bacteria.

CONCLUSIONS

There exist differences in chemical composition of each one of the extracts used in the antibacterial activity against the evaluated bacteria.

The plant extracts of sierrecilla, eucalyptus and oregano may inhibit the growth of *Avibacterium paragallinarum*, and *Pasteurella multocida*, causual agents in respiratory infections in chickens. They may be one possible alternative for the preventive treatment of respiratory infections.

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