



***In vitro* Analysis of Micro Emulsified Essential Oil against Pathogenic Strains of *Escherichia Coli*, *Salmonella Entritidis* and *Pasteurella Multocida* Isolated from Broilers**



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Abstract

The objective of the study is to investigate antimicrobial effect of the micro emulsified essential on the clinical isolates obtain from the poultry flock. To achieve this goal, clinical samples from the intestinal and respiratory tract of the broilers were collected. Three main pathogenic strains of organisms were isolated viz., *Escherichia coli*, *Salmonella enteritidis* and *Pasteurella multocida* which were stored in -20 °C until further study. MIC₅₀ of micro emulsified essential oils was determined by agar dilution method. The 50% inhibitory concentration for *S. enteritidis* is found to be 3.906µL/mL. 50% inhibitory concentration for enteric *P. multocida* and respiratory *P. multocida* is 3.906µL/mL. The MIC₅₀ for *E. coli* was less than 1.953µL/mL. Thus, we can conclude that essential oils in micro emulsified form have inhibitory action at therapeutic doses against *P. multocida*, *E. coli* and *S. enteritidis*.

Keywords: Herbofloxin; ORP; Poultry; Pathogens; Water

Introduction

Poultry is affected by the various diseases, especially flocks that have low immunity resulting in the significant economic losses due to high feed conversion ratio (FCR) and mortality [1]. They are also susceptible to secondary infections due to responses to irregular vaccination and immune suppression [2]. *Escherichia coli* are found to be one of the most important causes of economic loss to poultry production. However, ammonia, mycoplasmas, Newcastle disease virus (NDV), Infectious bronchitis virus (IBV) is considered to be the predisposing agents for *E. coli* dissemination. Pathogenic *E. coli*, *Salmonella* and *Pasteurella* strains are found in the intestinal tract of hatched chicks, suggesting rapid spread after hatching [3]. The Pathogenic and non-pathogenic strains can be transferred from feces to the litter and environment.

With the advancement of natural and synthetic drugs, it is now possible to treat several serious bacterial infections [4]. However, only 1/3rd of the known diseases have been treated using synthetic medicines [5], due to emergence of multiple drug resistant pathogens [6,7]. Despite the potential use of

medicinal plants for the treatment of many diseases from several centuries, its use in clinical microbiology is understudied [8]. Water forms one of the most important input for the livestock industry. The quality of the water mainly depends on the pH and oxido-reduction potential (ORP) because all major chemical and biological processes that happened in water are either directly influenced by or alter the pH and the ORP. Pathogens can be reduced by chlorinated water, however the effect of essential oils on the chlorinated water against the pathogens associated with poultry has not been studied. The phyto-constituents such as Eucalyptus and peppermint oils have been used to modulate immune system of birds [9]. The aim of the study is therefore, to determine the antimicrobial effect of the as a micro emulsified essential oils (Herbofloxin™) on the clinical isolates obtained from the poultry flock.

Materials and Method

Collection of clinical samples

The clinical sample from the intestine and respiratory tract of the poultry was collected using sterile swabs. Isolates from

poultry were from clinical and subclinical infections. The samples were forwarded to the Omega Laboratory for microbiological investigation.

Sample analysis

For the isolation of *Escherichia coli*, the swab was collected in sterile saline and loopful of the suspension was isolated on MacConkey agar (Difco) plates and incubated at 37 °C for 24h. The lactose-positive colonies were streaked on the Levine’s eosin-methylene blue (L-EMB) agar (Difco) from the MacConkey agar plates. Isolates were confirmed as *E. coli* by using IMViC test [10], including indole and methyl red tests, Voges-Proskauer (VP) test and the inability to grow on citrate agar.

For the isolation of *Salmonella enteritidis*, the swab was inoculated in 250mL conical flask containing 100mL Selenite cystine (SC) broth. The flask was incubated at 37 °C for 24h on a rotary shaker for proper mixing. Thereafter, a loopful of the culture was streaked on Xylose lysine desoxycholate (XLD) agar (Difco) and Bismuth sulfite (BS) agar (Difco). The isolates were confirmed as *S. enteritidis* by using Triple sugar iron agar (TSI) (Difco) slant and Lysine iron agar (LIA) (Difco) slant.

The identification and isolation of *Pasteurella multocida* was carried out as previously described [11]. The sample was collected in 2mL tryptophan broth and streaked on a selective medium Tryptose blood agar base (Difco) containing 5% citrated bovine blood, 1% neomycin and 0.02% bacitracin. After 24h incubation at 37 °C, suspected colonies of *P. multocida* were sub cultured. The isolates were confirmed to be *P. multocida* as previously described [12].

Table 1: Determination of Minimum Inhibitory Concentration (MIC50) by Agar dilution method.

Dilution	1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Herbofloxin concentration (µL/ mL)	1000	500	250	125	62.5	31.25	15.625	7.812	3.906	1.953
Herbofloxin	1.0mL	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Diluent	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
										
Inoculum (1.2X 10 ⁷ /mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Results and Discussion

The prevalence of antimicrobial resistance in a population is mostly associated with antibiotic consumption as selection and spread of these resistant bacteria have increased due to the pressure exerted by these antibiotics. As a result, resistance is the most common outcome of excessive use of antibiotics where appreciable host-to-host contact occurs [13]. In this case, resistant pathogens are easily disseminated via contaminated water, fecal contact or contaminated soil environment [14]. The main objective of this study is to determine inhibitory effect of micro emulsified essential oil (Herbofloxin) on the pathogens originating from the poultry flock.

To investigate the antimicrobial effect three main field bacteria viz., *E.coli*, *S. enteritidis* and *P. multocida* were selected.

All the bacterial isolates were isolated on plate count agar and incubated at 37 °C for 24h and thereafter maintained at -20 °C in nutrient broth with 50% (v/v) sterile glycerol.

Preparation of inoculum

The bacterial suspensions of the identified isolates were adjusted with sterile saline to a concentration of 1.2 X10⁷CFU/ml. The antibacterial assay was carried out by serial dilution method in order to determine the antibacterial activity of micro-emulsified essential oils against the pathogenic bacteria. The micro-emulsified essential oils known as Herbofloxin™ where sourced from Vinayak ingredients (india) pvt. ltd. It is marketed as a natural growth promoter.

Determination of minimum inhibitory concentration (MIC₅₀) by agar dilution method

The minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. It is the most widely used basic laboratory analysis of the activity of an antimicrobial agent against an organism. It is important in terms of diagnosis to corroborate resistance of microorganisms to an antimicrobial agent.

The minimum inhibitory concentrations (MIC₅₀) were performed by a serial dilution technique. The Herbofloxin (µL/mL) is diluted by a factor of 2 up to ten dilutions. The test organisms (1.2X10⁷CFU/ml) are added to the dilutions of Herbofloxin. A loopful of culture is spread plated on Mueller-Hinton media. Results were obtained after 24h of incubation at 37 °C. The procedure is summarized in Table 1.

To attain this goal, clinical samples from the infected intestine and respiratory tract of the poultry were collected. The pathogens were then isolated, identified and stored for further study. In the present study, the antimicrobial activity of Herbofloxin is determined using agar dilution method. The activity was analyzed quantitatively on the basis of Colony Forming Units. MIC50 of the pathogenic isolates were determined. The results revealed that 50% inhibitory concentration for *Salmonella enteritidis* is 3.906µL/mL. The MIC₅₀ for *Pasteurella multocida* isolated from intestine and respiratory tract is found to be 3.906µL/mL. *P.multocida* has high affinity for respiratory organs compared to gastrointestinal tract organs [15,16]. Although, respiratory tract and organs such as liver, colon and duodenum also hold high concentration of *P.multocida* [17,18]. This is because; large amount of blood is circulated into the liver

which contributes to the dissemination of bacteria. Further, gastrointestinal flora also competes and suppresses the growth of *P.multocida* within the intestine [19-21]. The MIC₅₀ for *E. coli* is considered less than 1.953µL/mL. The results are summarized in the Table 2 & Table 3. Hence, micro emulsified essential oil used has inhibitory action at therapeutic doses against bacteria like *E. coli*, *S. enteritidis* and *P. multocida*. Our results therefore, suggest that it can be used as bacteriostatic/bactericidal at 125 to 250mL/1000 Liter. In conclusion, emulsified essential oil contains potential antimicrobial components that may help to

prevent and treat various poultry diseases associated with *E.coli*, *S. enteritidis* and *P. multocida*. These pathogens are responsible for causing diarrhea, dehydration, weight loss and respiratory diseases, causing significant economic loss to poultry industry. The absence of such pathogens in the intestine leads to healthier gastrointestinal tract, which will not hinder the optimal growth of the villi. Therefore, longer villi will provide more surface area for better absorption of nutrients. This will then lead to a more efficient feed conversion and rapid growth, which results in better profits for the producer.

Table 2: Determination of MIC50 of Herbofloxin on different clinical isolates.

Concentration of Herbofloxin (µL/mL)	Dilution	Colony Forming Units (CFU)/mL			
		<i>E.coli</i>	<i>S. enteritidis</i>	<i>Enteric P. multocida</i>	<i>P. multocida</i>
1000	1	0	0	0	0
500	1:02	2.0x10 ²	2.0x10 ²	5.0x10 ²	1.2x10 ³
250	1:04	7.0x10 ²	4.0x10 ²	9.0x10 ²	2.2x10 ³
125	1:8	9.0x10 ²	8.0x10 ²	1.3x10 ³	3.4x10 ³
62.5	1:16	1.9x10 ³	2.2x10 ³	2.6x10 ³	4.2x10 ³
31.25	1:32	2.3x10 ³	3.5x10 ³	4.2x10 ³	5.5x10 ³
15.625	1:64	3.0x10 ³	4.5x10 ³	5.9x10 ³	7.1x10 ³
7.812	1:128	4.5x10 ³	5.2x10 ³	6.9x10 ³	8.2x10 ³
3.906	1:256	6.0x10 ³	6.8x10 ³	8.8x10 ³	9.0x10 ³
1.953	1:512	8.2x10 ³	9.2x10 ³	1.21x10 ⁴	1.44x10 ⁴

Table 3: Determination of Colony Forming Units (CFU)/mL of different clinical isolates.

Culture Untreated with Herbofloxin	Colony Forming Units (CFU)/MI
<i>E.coli</i>	1.86x10 ⁴
<i>S. enteritidis</i>	1.67x10 ⁴
<i>Enteric P. multocida</i>	1.70x10 ⁴
<i>P. multocida</i>	1.82x10 ⁴

The broiler performance for both the treatments were observed that have water with ORP values close to 750 millivolts (Table 4). Lower values up to 250 millivolts indicate presence of heavy organic compounds that has not been properly disinfected by chlorine [22], whereas, the ORP value of 650 millivolts or greater indicates good quality water [23]. In contrast to these reports, using negative ORP water used in water had its own benefits [24]. These reports suggest that using oxidizing agents with positive high ORP may in fact be unhealthy to the birds and Thus, the micro emulsified essential oils used as antimicrobial and a disinfectant without affecting the ORP values of the water warrants further study [25-27].

Table 4: ORP values of the treatments.

	Control (Chlorinated)	Treatment (Herbofloxin™)
Water ORP values	754	254

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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