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In Vitro Evaluation of Antimicrobial Activities of *Eugenia caryophyllata* and *Origanum vulgare* Against Avian *Escherichia coli* Isolates

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ABSTRACT

Background**:** The use of conventional antibiotics to prevent this infection is a usual in the poultry industry, specially to reduce the mortality associated with colibacillosis. Essential oils have demonstrated broad spectrums in terms of antimicrobial activities against various pathogens, *Escherichia coli* among them. The aim of this study was to evaluate the *in vitro* susceptibility of avian *E. coli* strains to *Eugenia caryophyllata* (clove) and *Origanum vulgare* (oregano) essential oils, at three different concentrations (2, 5, and 8%), and to the conventional antimicrobials, gentamicin and nalidixic acid.
Materials, Methods & Results**:** Seventeen strains of *Escherichia coli* isolated from broiler bedding were tested and a standard *E. coli* strain (ATCC 25922) was used as a susceptibility test control. The antimicrobial activity of plant extracts from Eugenia caryophyllata and *Origanum vulgare*, obtained commercially from Laszlo Aromatologia Ltda., was tested in three concentrations (2, 5 and 8%) using the method of disk diffusion on agar according to the M2 - A8 protocol (CLSI, 2003) adapted to plant extracts and to the conventional antimicrobials gentamicin (10 µg) and nalidixic acid (30 µg), commercially obtained from Oxoid. The results showed that the extract from *Origanum vulgare* had an inhibitory activity on *E. coli* regardless of its concentration whereas the extract from *Eugenia caryophyllata* showed antimicrobial activity in 29.4% (5/17) of the isolated strains only at the concentration of 8%. All avian isolates were sensitive to gentamicin (100% of the strains tested); however, only 23.5% (4/17) of the samples were sensitive to nalidixic acid.
Discussion**:** The impressive antimicrobial activity of the *Origanum vulgare* against *E. coli* avian origin was confirmed in previous studies against both Gram-positive and Gram-negative bacteria. The essential oil of *Origanum vulgare* was more antimicrobial activity that *Eugenia caryophyllata*. Thymol and carvacrol is main antimicrobial component of the essential oil of *Origanum vulgare*, being responsible for the distortion to the physical structure of cells, leading to destabilization of the cell membrane, changing its permeability and denaturing essential enzymes. As for, the major constituent of the essential oil of *Eugenia caryophyllata* is eugenol, and their pharmacological effects depend on the concentration of free eugenol in living tissue, which may account for the better antimicrobial activity in the treatment with 8% of this extract. All the strains tested in this study were sensitive to gentamicin, while most of them (80%) were resistant to nalidixic acid. The gentamicin has the highest spectrum of action among aminoglycoside, however, bacteria rapidly acquire plasmid-mediated resistance to this ingredient in the presence of selective pressure. The beginning of antimicrobial resistance by zoonotic bacteria has important implications for public health. Data from several researchers suggest that improper selection and disorderly use of conventional antimicrobials can lead to the resistance of various pathogens and, consequently, their access to consumers through foodstuffs. Therefore, the use of *Origanum vulgare* oil extract was found to be effective on avian *E. coli* strains and may be an alternative for reducing the losses caused by these bacteria in poultry production whenever a limitation in the use of traditional antibiotics exist.

Keywords: antimicrobial resistance, essential oils, *Escherichia coli*, poultry.

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INTRODUCTION

The use of conventional antibiotics to prevent this infection is a usual in the poultry industry [41], specially to reduce the mortality associated with colibacillosis [7,21]. The European Union has banned the use of conventional antimicrobials as growth promoters in the feeds of farming animals in 2006 [14], based on the principle that bacterial resistance to many antimicrobials have increased [10,15].

The use of plant extracts as growth promoters or preventive/therapeutic agents is still recent; however, the number of surveys is gradually increasing due to various factors, such as bacterial resistance to some antibiotics or even the demand for therapeutic treatments in alternative production systems [10].

Essential oils have demonstrated broad spectrums in terms of antimicrobial activities against various pathogens [2,29], *E. coli* among them [8,18,38]. The extract from *Origanum vulgare* has antibacterial and antifungal activities against different pathogens that are attributed to phenolic compounds, such as carvacrol and thymol, which they have in large quantities [2,25]. Another extract of interest, which stands out for its antimicrobial properties is the *Eugenia caryophyllata* that containing 90% of active principle eugenol, which is known for its action anesthetic, local analgesic, and anti-inflammatory power and for having antibacterial effects [12].

The aim of this study was to evaluate the *in vitro* susceptibility of avian *Escherichia coli* strains to *Eugenia caryophyllata* (clove) and *Origanum vulgare* (oregano) essential oils, at three different concentrations (2, 5, and 8%), and to the conventional antimicrobials, gentamicin and nalidixic acid.

MATERIALS AND METHODS

Microorganisms

Seventeen strains of *Escherichia coli* were isolated from chicken litter and stored at -20°C in Brain Heart Infusion broth (BHI)[1] with addition of 20% glycerol at the Center for Diagnosis and Research in Avian Pathology of the Federal University of Rio Grande do Sul (UFRGS-CDPA). One strain of *E. coli* ATCC 25922 (American Type Culture Collection)[2], was used as a positive control.

Conventional antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed using the disk diffusion method on agar according to the M2 - A8 protocol (CLSI 2003), and the antimicrobials gentamicin (10 µg) and nalidixic acid (30 µg), commercially obtained from Oxoid[3], were used.

Plant Extracts

The antimicrobial activity of plant extracts was tested using the method of disk diffusion on agar according to the M2 - A8 protocol (CLSI, 2003) adapted to plant extracts. Extracts from *Eugenia caryophyllata* (clove) and *Origanum vulgare* (oregano) were obtained by steam distillation and obtained commercially from Laszlo Aromatologia Ltda[4]. The chemical identification of the main components was provided by the company through gas chromatography analysis (Table 1). For the disk diffusion technique, the extracts were diluted in 10% ethanol solution at concentrations of 2, 5, and 8%, stored in amber bottles and sealed with parafilm.

Inoculum growth method

Samples were removed from the glycerol stocks, enriched in 5 mL of Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h according to recommendations from document M2 - A8 [6]. Each sample was inoculated by plating on Tryptone Soya Agar (TSA)[3], and incubated for 24 h at 37°C. Once the period of bacterial growth elapsed, 3 to 5 CFUs were inoculated in 5 mL of 0.9% saline solution with a 0.5 McFarland turbidity standard, corresponding approximately to 1.0-2.0 x 10^8 CFU.mL^{-1} of *E. coli* (ATCC 25922). The number of colonies was confirmed and then introduced a sterile swab in the bacterial suspension for three m. The suspension was seeded onto the surface of a Muller-Hinton agar plate (MHA)[1], towards 3 directions until a uniform smear was achieved. After the inoculum was dried, sterile filter paper discs without reagent[3], 6 mm in diameter on agar and were soaked with 10 µL of each essential oil, diluted to the three concentrations studied (2, 5, and 8%). Discs of gentamicin and nalidixic acid were applied onto different Mueller-Hinton agar plates [34]. The distance between discs was calculated to avoid overlap of the inhibition zones. Readings were taken after 24 h of incubation at 37°C by measuring the inhibition halos of bacterial. All tests were performed in duplicates.

Statistical analysis

The normality and heteroscedasticity assumptions were tested using the Shapiro and Levene tests, respectively. In both cases, the null hypothesis was rejected. The relation difference between independent treatments was tested using the Kruskal Wallis test (nonparametric analysis of variance - ANOVA) and were established using the Wilcoxon-Mann-Whitney multiple comparisons test. The level of significance was set at $P < 0.05$. All analyses were performed using the Minitab statistical analysis program (Minitab 15.1)[5].

Table 1. Chemical composition of *Origanum vulgare* and *Eugenia caryophyllata* essential oils (Laszlo Aromatologia Ltda.).

Botanical name	Family	Common name	Composition	(%)
Origanum vulgare	Lamiaceae	Oregano	α-pinene	0.5
			Camphene	1.1
			p-cymene	4.0
			Limonene	1.4
			1,8-cineol	0.7
			Phellandrene	1.2
			γ-terpinene	3.2
			Camphor	0.7
			Terpinen-4ol	0.8
			α-terpineol	0.2
			Thymol	4.3
			Carvacrol	76.5
			Caryophyllene	1.5
Eugenia caryophyllata	Myrtaceae	Clove	Eugenol	61.2
			β-caryophyllene	31.2
			Eugenyl acetate	3.4

RESULTS

Significant differences were found between the effects of the different treatments against *E. coli* strains when results of the multiple comparisons between the diameters of inhibition halos were considered ($P < 0.001$) [Table 2].

Data revealed that the plant extracts and conventional antimicrobials tested showed inhibitory *E. coli* activity at varying degrees. The oregano extract showed antibacterial activity against all the strains analyzed, including the indicator strain ATCC 25922. The mean diameter of the inhibition halo at the different concentrations were 11.9 ± 1.2 mm at 8%, 10.5 ± 1.1 mm at 5% and 9.3 ± 0.8 mm at 2%. Therefore, an increased direct relationship between the concentrations of the oregano extract the inhibitory activity on the growth of *E. coli* strains was observed, as illustrated in Figure 1.

The extract from clove at the concentrations of 2 and 5% showed no antimicrobial activity. The only activity of this extracts was found at 8% concentration and only in 29.4% of the strains (5/16). A mean inhibition halo diameter of 7.9 ± 0.8 mm (Figure 2) was found. Thus, the oregano showed a broader action spectrum for presenting a halo diameter of 13.5 mm.

All isolated strains were sensitive to gentamicin; however, when the activity of nalidixic acid was tested, only 23.5% of the specimens (4/16) were sensitive, indicating the existence of *E. coli* strains that were resistant to this conventional antimicrobial. Although both essential oils tested showed antimicrobial activity in *E. coli* isolates, the tests with gentamicin had the largest bacterial inhibition halos, with values up to 32.5 mm, which is more than twice the diameter found when oregano was tested at its highest concentration (8%).

Table 2. Diameters of inhibition (mm) from avian *E. coli* isolates after *in vitro* testing with *Origanum vulgare*, *Eugenia caryophyllata* and conventional antimicrobials (Gentamicin and nalidixic acid).

	Treatments Antimicrobials							
Strain	*Origanum vulgare* (%)			*Eugenia caryophyllata* (%)			Antimicrobial	
E.coli	2	5	8	2	5	8	Nalid Ac.	Genta
	(Halo´s Diameter in millimeters)							
ATCC	10	11.5	12.5	NI	NI	NI	16	29
5	9	10	11	NI	NI	NI	21.5	25.5
8	8.5	9	12	NI	NI	NI	NI	31.5
9	9	11	12.5	NI	NI	8	NI	26
11	10	11	12.5	NI	NI	7	NI	25
14	9	10	10.5	NI	NI	8	NI	21
19	9	10	11	NI	NI	NI	NI	25.5
25	8	9	11	NI	NI	NI	NI	32.5
26	9	10	11	NI	NI	NI	NI	15
31	8.5	9.5	10	NI	NI	7.5	NI	23.5
39	10	11.5	13.5	NI	NI	NI	NI	28.5
56	9.5	11	13.5	NI	NI	9	NI	24
77	9	9	10	NI	NI	NI	NI	18.5
79	10	12	13.5	NI	NI	NI	NI	20
144	11	11.5	12.5	NI	NI	NI	NI	22.5
159	8	9.5	11	NI	NI	NI	NI	20
178	10	12	12	NI	NI	NI	12.5	24.5
272	9.5	12	13.5	NI	NI	NI	21.5	25.5
*M ± SD	9.3 ± 0.8^{bde}	10.5 ± 1.1^{cd}	11.9 ± 1.2^{cd}	0 ± 0^{a}	0 ± 0^{a}	7.9 ± 0.8^{ae}	17.8 ± 4.4^{ab}	24.3 ± 4.5^{c}

NI: No inhibition; M: mean; SD: standard deviation; Nalid. Ac.: nalidixic acid; Genta: gentamicin. Number of samples in each treatment = 17+standard (ATCC). [abcde]Equal letters in the same line statistically differ with a 95% confidence interval. *Although the mean is shown, the statistical test performed multiple comparisons of the mean ranks in each treatment.

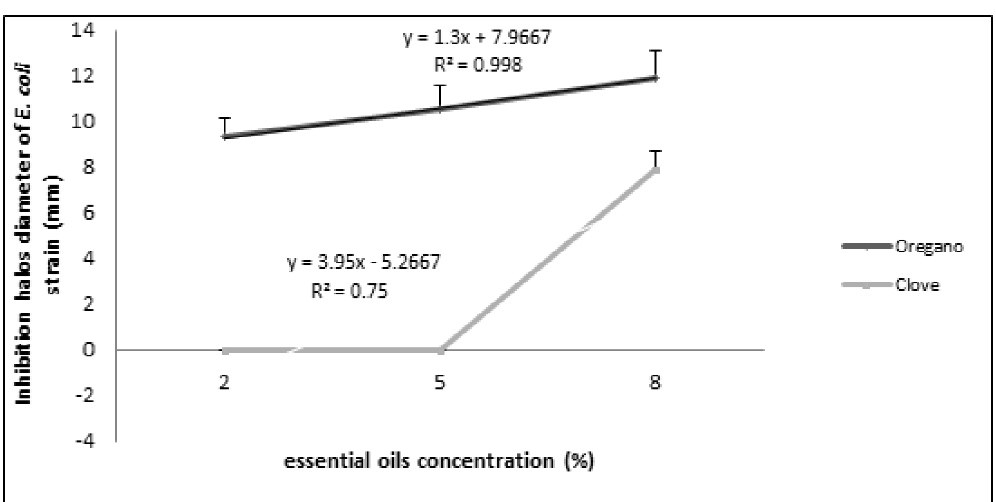

Figure 1. Average behavior of inhibition halos (mm) for avian *E. coli* strains after *in vitro* testing with the extracts of *Origanum vulgare* (oregano) and *Eugenia caryophyllata* (clove) at different concentrations (2, 5, and 8%).

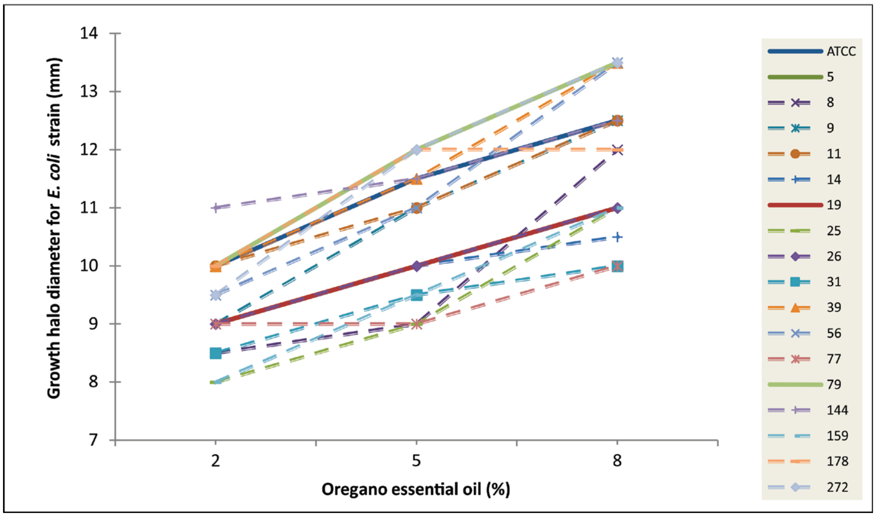

Figure 2. Behavior of inhibition halos (mm) of each avian *E. coli* strain after *in vitro* testing with the extract of *Origanum vulgare* (oregano) or *Eugenia caryophyllata* (clove) at different concentrations (2, 5, and 8%).

DISCUSSION

As noted in this study, the extracts from *Origanum vulgare* showed better results when compared to those from *Eugenia caryophyllata*. These results are in agreement with other reports which found similar results when *Staphylococcus aureus*, *E. coli* and Salmonella. However, both extracts showed activity against all bacteria examined, but the extract from oregano showed the largest diameter of bacterial inhibition (26.7 mm and 29.3 mm for *E. coli* and *S. aureus*, respectively) [34].

The chemicals thymol and carvacrol cause distortion to the physical structure of cells, leading to their expansion those results in destabilization of the cell membrane, changing its permeability and denaturing essential enzymes. Furthermore, they cause variations in pH and in the electrical potential of cells changing their proton-motive force [2]. The bactericidal activity of plant extracts vary depending on the concentrations of the existing constituents, which may also depend on abiotic factors, such as soil type, climate, agricultural practices, oregano variety, and extraction process [9,11,26].

Additionally, previous research have shown that the antimicrobial activity of the *Origanum vulgare* extracts against the growth of pathogenic bacteria (*S. aureus*, *E. coli* and *Salmonella choleraesius*) using the disk diffusion method and found that *Salmonella choleraesius* showed a greater halo inhibition (29.0 ± 0.08 mm) than the other bacteria tested [3]. Effectiveness of the oregano extract is primarily on Gram-positive bacteria, particularly *S. epidermidis*, while only *E. coli* was inhibited among Gram-negative bacteria [22]. Oregano extracts also showed prominent activity against ESBL (Extended Spectrum β-Lactamase) - producing *E. coli*, a strain that is resistant to multiple antibacterial agents [13]. It seems that the Gram-negative species are less susceptible to the action of plant extracts from *Origanum vulgare* and *Eugenia caryophyllata* due to the existence of an outer membrane surrounding the cell wall, which restricts the diffusion of hydrophobic compounds through the outer wall [2].

Results obtained showed that the essential oil of *Eugenia caryophyllata* had less antimicrobial active than the tested samples in relation to the *Origanum vulgare*. The major constituents in the *Eugenia caryophyllata* extracts found 78% of eugenol [12,34] and 8% of eugenyl acetate [26]. Those extracts seem to be potent antimicrobial agents capable of degenerating proteins, but at low concentrations they tend to stabilize cell membranes [44]. Accordingly, its pharmacological effects depend on the concentration of free eugenol in living tissue, which may account for the better antimicrobial activity in this study, in the treatment with 8% of this extract.

All the strains tested in this study were sensitive to gentamicin, while most of them (80%) were resistant to nalidixic acid. The emergence of antimicrobial resistance by zoonotic bacteria has important implications for public health. Data from several researchers suggest that improper selection and disorderly use of conventional antimicrobials can lead to the resistance of various pathogens and, consequently, their access to consumers through foodstuffs [30]. The high antimicrobial resistance to nalidixic acid in avian *E. coli* isolates found in the present study was previously reported. Samples of *E. coli* isolated from pigs and poultry in China that were characterized in terms of virulence genes, susceptibility to antimicrobials, and specific mechanisms of resistance to fluoroquinolones, were 100% resistant to nalidixic acid, but also showed to be resistant to tetracycline, sulfamethoxazole, ampicillin and sulfamethoxazole associated to trimethoprim, suggesting that multiple resistances in *E. coli* isolates include genetic variants resistant to fluoroquinolones [41].

Studies conducted to evaluate the susceptibility of *E. coli* strains isolated from broiler chickens with colibacillosis [21] reported that nalidixic acid showed a high proportion of resistant specimens, however, [23], using ciprofloxacin, norfloxacin and gentamicin observed no resistant strains with norfloxacin and gentamicin being fully efficient and without resistance [21].

Furthermore, previous articles when analyzed the antimicrobial resistance profiles of *E. coli* isolates from fecal samples [20] and colibacillosis above isolated [21] of broiler chickens, demonstrated that all isolates were sensitive to gentamicin. This finding is probably due to the limited use of this antimicrobial in poultry production [5]. However, results differing from those found in this study show rates of sensitivity to gentamicin of 71% in *E. coli* samples isolated from cellulitis lesions in broilers [4] and 97.1% [32] in isolates from chickens diagnosed with colibacillosis. The gentamicin has the greatest spectrum of action among aminoglycosides; however, bacteria rapidly acquire plasmid-mediated resistance to this ingredient in the presence of selective pressure [42].

CONCLUSIONS

In view of the results observed, it can be concluded that the extracts from *Origanum vulgare* and the antimicrobial gentamicin showed bactericidal activity against *Escherichia coli* strains isolated from broiler chickens, demonstrating to be effective drugs when evaluation was done *in vitro*. The essential oil of *Eugenia caryophyllata* demonstrated lower antimicrobial activity and only when added at the concentration of 8%. Nalidixic acid inhibited 4 of 17 *E. coli* strains only, showing a high resistance rate. Essential oils may be alternatives to the use of growth promoters in poultry; however, a general statement for the activity of all essential oils as antimicrobials is not possible since their chemical composition is to variable.

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