



The Effects of some Herbal Essential Oils against *Salmonella* and *Escherichia coli* Isolated from Infected Broiler Flocks

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ABSTRACT

Escherichia coli and *Salmonella* spp are two bacterial infectious diseases responsible for heavy economic losses in the poultry industry. The emergence of antimicrobial resistance and its potential harmful threat to human health has led to a need to find safe alternatives for the control of these bacteria. To this end, the use of herbal remedies in poultry has been suggested. In this study, we have investigated the effect of essential oils extracted from five different herbal plants against *Salmonella* spp and *Escherichia coli* that have been isolated directly from infected broiler flocks. Standard Disk-diffusion method, Minimum Inhibition Concentration and minimum bactericidal concentration were used to determine the inhibitory effect of these essential oils. Also, tetracycline was used as a control group. Among the essential oils, *Carum copticum* had the highest antibacterial properties. The maximum inhibition zone in diameter against *Salmonella* and *Escherichia coli* were respectively 26.7 and 22.5 mm that concern about *Carum copticum* essential oils. According to the results of this study, it was found that some of the essential oils have a stronger antibacterial effect than tetracycline. So, after the complementary studies, some of these herbal plants can be suggested as alternatives to antibiotics for treating infections caused by these bacteria in poultry industry.

Keywords: Essential oil, Herbal plant, *Escherichia coli*, *Salmonella*

INTRODUCTION

The use of antibiotics has improved poultry performance effectively and economically however there has been a developing controversy and much criticism surrounding the use of antibiotics as growth promoters in the poultry industry (Abd El-Galil and Mahmoud, 2015). The high incidence and rising frequency of antibiotic resistance among the bacteria populating poultry presents many public health issues. Based on the theories suggesting that pathogenic bacteria have the ability to become resistant to specific antibiotics, it is difficult to develop drugs and treatments with the abilities to kill them (Hoffman-Pennesi and Wu, 2010). These antibiotic resistant bacteria can be transmitted from poultry to humans through the food chain with serious consequences on public health (Abiala et al., 2016). It is

generally accepted that enteric infection and its associated gastrointestinal dysfunction is the major stress that impairs intestinal function and compromises the immunity of food-producing poultry, therefore leading to growth retardation and increased morbidity and mortality of poultry. Strategies including the use of enzymes, organic acids, probiotics, prebiotics, and of the implication of herbal plants for controlling enteric infection, improving poultry immunity, and ameliorating intestinal function are common approaches in order to reduce and maximize poultry production (Gong et al., 2013). Nowadays, herbal plants are used on a large-scale in medicines against drug-resistant bacteria, which are considered as one of the most important reasons for the lack of success of treatment in infectious diseases. Herbal plants are a major source of new medicines and may be considered as an alternative to the usual drugs (Al-Mariri

et al., 2014). Traditionally, many plant extracts and oils are used as medicinal plants in Iran for many purposes, particularly for respiratory and gastrointestinal disorders (Feiz Haddad *et al.*, 2017). The essential oils of 5 herbal plants used in this work include *Pulicaria gnaphalodes* L, *Ducrosia anethifolia* L, *Carum copticum* Benth L, *Foeniculum vulgare* Mill and *Majorana hortensis* Minch L (Habibi *et al.*, 2017). Essential oils can be beneficial as a feed additive to promote the gut health of chickens and help to reduce the risk of bacterial infections. As consumers are trending toward more health-conscious eating and natural alternatives instead of artificial products, essential oils obtained from herbal plants can be used as natural feed additive for poultry (Hoffman-Pennesi and Wu, 2010). The aim of this study was to screen the *in vitro* antibacterial activity of 5 plants essential oils against two gram-negative bacteria including *Escherichia coli* (*E. coli*) and *Salmonella* spp that were isolated from broiler flocks, were then compared to common antibiotics used in the poultry industry.

MATERIALS AND METHODS

Collection of herbal samples

The various medicinal plants were obtained from the mountainous area in south of Iran between December 2016 and May 2017. The plants include: *Pulicaria gnaphalodes* L, *Ducrosia anethifolia* L, *Carum copticum* Benth L, *Foeniculum vulgare* Mill and *Majorana hortensis* Minch.

Plants essential oil

In order to provide essential oils, 100 g of each plant (*P. gnaphalodes* L, *D. anethifolia* L, *C. copticum*, *F. vulgare* Mill and *M. hortensis*) was introduced in the distillation flask (1 L), which was connected to a steam generator via a glass tube and to a condenser to retrieve the oil. Aromatic molecules of the essential oils were released from the plant material and evaporated into the hot steam. The hot steam forced the plant material to release the essential oils without burning the plant material itself. Then, steam containing the essential oils was passed through a cooling system in order to condense the steam. The steam was applied for 3 hours. Afterward, the essential oils were collected in tightened vials and stored in a refrigerator. For the carried out of antimicrobial activity test, use of suitable chemical solution, therefore the essential oils were diluted to 100

mg mL⁻¹ in dimethyl sulfoxide (DMSO) (Habibi *et al.*, 2017).

Isolation of bacteria

Samples were collected over a period of three weeks from four poultry farms in the south of Iran. Two of these samples showed signs of colibacillosis such as mucous nasal discharge, sneezing, conjunctivitis, facial swelling, Perihepatitis, Peritonitis and Cellulitis over the abdomen. Two other samples showed salmonellosis symptom including depression, ruffled feathers, closed eyes, white diarrhea, vent pasting, loss of appetite, intestinal inflammation and unabsorbed yolk sacs. The sampling in colibacillosis was obtained from the liver and air sack swaps. But the samples in salmonellosis were from cloacal swaps.

Identification of bacteria

Cotton swabs were moistened with autoclaved and placed in sterile bags prior to use in the processing plant. Swabs sampling has done from ventral cloaca, cecum, air sace and trachea that approximately 30 s using a vigorous back and forth motion. The swabs were placed in a tube containing a medium suitable for bacterial transport (Transwab; Medical Wire and Equipment Co. Ltd., Corsham, England) and were sent to the laboratory by ordinary mail. On arrival, the 10 swabs were pooled in a tube containing 3 ml of sterile water. The swabs were whirl-mixed in the tube and were left for approximately 5 min sat room temperature to release the bacteria. For the isolation of *E. coli*, 200 µl of above solution was cultured onto MAC plates and incubated at 35°C for 24 h. Following incubation, lactose-positive colonies (3-5 coloni) were streaked onto eosin-methylene blue agar plates. Typical *E. coli* colonies on eosin-methylene blue agar (green and shiny or with dark or purple centers) were subcultured in 10 ml of Trypticase soy broth and were incubated for 24 h at 37°C. The broth cultures were tested for indole production, and indole-positive cultures were confirmed to be *E. coli* by using API 20E (Biomerieux Vitek, Inc., Hazelwood, Mo). To isolate *Salmonella*, 200 µl of *salmonella* suspicious solution was mixed with the same volume of double-concentrated lactose broth. After incubation at 35°C for 24 h, 1.0 ml of the enrichment broth was transferred into 9.0 ml of tetrathionate broth and incubated at 42°C for 24 h. Following 24 h of incubation, the broth culture was streaked onto xylose-lysine-tergitol 4 agar plates and incubated for 24 h at 37°C. Presumptive *Salmonella*

colonies (3-5 coloni) on xylose-lysine-tergitol 4 plate were selected and used to inoculate triple sugar iron slants, which were then incubated for 24 hrs at 37°C. The identities of *Salmonella* isolates were confirmed by the use of the oxidase test and biochemical strips (API20E, BioMerieux) (Drobniewski, 1993).

Antimicrobial assay

Agar gel disk diffusion test (qualitative method) and Minimum Inhibitory Concentration (MIC) as well as Minimum Bactericidal Concentration (MBC) were used in this study.

Disc diffusion susceptibility

Antibacterial susceptibility assay Muller-Hinton Broth (MHB, Merck) medium was used to grow the test isolates for 22 h at 37°C. Final bacterial numbers were standardized to 1×10^6 cfu/ml. A total of 0.1 ml of bacterial suspension was poured into each plate, containing MHA. The surface culture was prepared by sterile L shape pipet pastor and allowed to remain in contact for 1 min. Thereafter, a 5% concentration of each plant extract and the essential oils was prepared. The sterile filter paper discs (6-mm diameter) were placed on the cultures, and 24 h after incubation at 37°C, the inhibition zone was measured in mm. Tetracycline was used as positive control standard, to determine the sensitivity of each bacterial species tested. All the tests were performed in triplicate (Karami et al, 2017).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

For each extract and essential oil a set of 9 sterile test tubes were used. The stock solutions (500 mg/ml) were further diluted in a 2-fold serial dilution to obtain the following concentrations: 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.91, 1.95, and 0.98 mg/ml. One test tube as a negative control and tetracycline as positive control were used. An aliquot of 1 ml of the bacterial suspension was inoculated into each tube. The negative control tubes were inoculated with the same quantity of extracts. All tubes were incubated at 37 C for 24 hours. The lowest concentration that did not permit any visible growth when compared with the control was considered as the MIC. The contents of all the tubes that showed no visible growth were cultured on MHA, incubated at 37 C for 24 hrs. The MIC was considered as the lowest concentration that could not produce a single bacterial colony and the MBC was defined as the lowest concentration of the

extract at which 99.9% of the inoculated microorganisms were killed (Aboaba et al., 2006).

Statistical analysis

The data was analyzed with Statistical Package for the Social Sciences (SPSS), version 16.0 software. All bacterial counts were converted to log₁₀ cfu/ml (g) for analysis and ANOVA was performed. Statistical significance was set at a P-value of $P \leq 0.05$.

RESULTS

In the present study, antibacterial activity of *Pulicaria gnaphalodes* L, *Ducrosia anethifolia* L, *Carum copticum* Benth L, *Foeniculum vulgre* Mill L and *Majorana hortensis* Minch L essential oils were recorded against *salmonella* spp and *E. coli* isolated from broiler flocks. For the evaluation of bacterial susceptibility to herbal agents, we carried out three standard tests including disc diffusion assay, MIC and MBC. Based on the results summarized in Table 1, essential oils from leaves of different herbal plants showed potential activity against *E. coli* that were isolated from broiler flocks with the mean zone of inhibition ranging between 10-26.7 mm. The results, as seen in Table 1, shows that the *E. coli* was the most susceptible to essential oil obtained from *Carum copticum* Benth with an inhibition zone range of 26.7 mm in diameter that was more than tetracycline with inhibition zone of 22 mm in diameter. The lowest effect of essential oil against *E. coli* was related to *Pulicaria gnaphalodes*, with 10 mm of inhibition zone. The activities of the 5 essential oils of herbal plants showed that *Carum copticum* Benth have the highest inhibition zone diameter against *salmonella* spp, and the second susceptibility of *salmonella* spp was related to *Majorana hortensis* Minch essential oil but the other essential oil had no effect on *salmonella* spp (Table 1).

The inhibition zone in diameter of *Carum copticum* Benth (22.5 mm) was more than the control positive agent (tetracycline). The MIC and MBC of essential oils at the concentrations range from 250 mg/ml to 0.98 mg/ml compared with the activity of tetracycline are shown in Table 2. In general, *E. coli* was more susceptible than *salmonella* spp to herbal agents. The results of the MBC method indicated that three herbal plants had antibacterial activity against *Salmonella* spp but *E. coli* was susceptible toward each five herbal plant agents (Table 2).

Table 1. Evaluation of inhibitory effects of plant essential oils using disc diffusion method (mm)

Bacteria	<i>P.gnaphalodes</i>	<i>D.anethifolia</i>	<i>F.Vulgare</i>	<i>M.hortensis</i>	<i>C.copticum</i>	Tetracycline	Control negative
<i>Salmonella</i> spp	0	0	0	18±2.8 ^{cd}	22.5±4 ^{*e}	18.5±2 ^{cd}	0
<i>E. coli</i>	10 ^a	11±2.3 ^a	14.7±3 ^b	16.5±3.8 ^{bc}	26.7±3.8 ^f	20±4 ^{de}	0

P.gnaphalodes: *Pulicaria gnaphalodes*, *D.anethifolia*: *Ducrosia anethifolia*, *F.Vulgare*: *Foeniculum vulgare*, *C.copticum*: *Carum copticum*. The different superscripts are significantly different ($P < 0.05$), *Mean ± standard deviation

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/ml) values of essential oils of the selected plants against isolated bacteria

Herbal Essential Oil	<i>Salmonella</i> spp		<i>E. coli</i> spp	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
<i>P.gnaphalodes</i>	125	NO	125	250
<i>D.anethifolia</i>	62.5	NO	7.8125	15.625
<i>F.Vulgre</i> Mill	62.5	250	3.91	15.625
<i>M.hortensis</i>	3.91	7.8125	3.91	7.8125
<i>C.copticum</i>	1.95	3.91	0.98	1.95
Tetracycline	1.95	3.91	0.98	3.91
Control negative	NO	NO	NO	NO

NO; No effect. *P.gnaphalodes*: *Pulicaria gnaphalodes*, *D.anethifolia*: *Ducrosia anethifolia*, *F.Vulgare*: *Foeniculum vulgare*, *C.copticum*: *Carum copticum*.

DISCUSSION

In populations, the alarming prevalence of antimicrobial resistance is a result of antibiotic consumption and because of the pressure exerted by these antibiotics, the spread of these resistant bacteria has increased (Dayaram et al., 2017). This is an indication that the indiscriminate use of conventional antimicrobials in the livestock and poultry industries has led to a steady increase in the antibiotic resistance. One of the most important reasons for the unusual use of antibiotics in the poultry industry is the presence of bacterial infections such as salmonellosis and colibacillosis which are caused by different isolates of *salmonella* and *E. coli*, respectively. Avian colibacillosis and salmonellosis are considered to be the major bacterial diseases in the poultry industry worldwide that are communicable to humans (Lutful Kabir, 2010). Both of these diseases are considered as the most important causes of severe financial loss by its association with high mortality and performance losses in the broiler industry. Therefore, researchers are trying to find out alternatives for antibiotics in order to control and treat these bacterial diseases. Herbal Plants have been documented as one of the sources that possess antimicrobial traits which are

chiefly synthesized during secondary metabolism. Plant based antimicrobial compounds have great therapeutic potentials as they can serve the purpose without any side effects associated with synthetic drugs (El-Mahmood and Doughari, 2008). In the current study, essential oils obtained from five herbal plants were used against two pathogenic bacteria. *Carum copticum* Benth essential oil was found to have the most effective antimicrobial property on *E. coli* among all the tested essential oils. Gas chromatography analysis of the essential oils from Iranian *Carum copticum* shows that the three most important constituents of these oils include Thymol, terpinolene and o-cymene (Mohagheghzadeh et al., 2007). Thymol and cymene are two potential antimicrobial agents that exist in *Carum copticum* Benth essential oil. The result of the disc's antibacterial susceptibility, MIC and MBC testing showed that both of the pathogen bacteria are highly susceptible to *Carum copticum* Benth essential oil. Previous studies have reported that the strong antimicrobial potential of the *Carum copticum* Benth can be attributed to thymol and its precursors, cymene and terpinene, have strong antimicrobial activities (Marino et al., 1999; Hassan et al., 2016). Based on current evidence, Ajowan EO can inhibit food-borne pathogenic microorganisms such as

Staphylococcus aureus (Vazirzadeh et al, 2013). The antimicrobial activity of thymol may be induced via modification of the cell membrane permeability and leakage of intracellular material. P-cymene, a major compound detected in *Carum copticum* oil, is a hydrophobic molecule and causes swelling of the cytoplasmic membrane (Burt, 2004). The antimicrobial potential of thymol, p-cymene, Carvacrol, and γ -terpinene against *E. coli* and *Staphylococcus aureus* has been reported in literature reviews (Cristani et al., 2007; Hassan et al., 2016). The results of our study are in consistent with the results of other researchers that have indicated the antimicrobial potential of *Carum copticum* oil against, *Escherichia coli*, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Klebsiella* spp, *Proteus vulgaris* and *Salmonella typhimurium* (Singh et al., 2002; Goudarzi et al., 2011; Hassan et al., 2016). *Majorana hortensis* has been used since times immemorial to treat a wide range of infections. It has been subjected to quite extensive phytochemical, experimental and clinical investigations. The results of this study show that *salmonella* spp and *Escherichia coli* are susceptible to the action of *Majorana hortensis* Minch essential oil. The inhibition zone of *Majorana hortensis* Minch essential oil against both of these bacteria were less than tetracycline, but *salmonella* was more sensitive to marijuana than *Escherichia coli*. In the previous studies, the antibacterial effect of this plant on *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Shigella boydii* was determined (Habibi et al, 2018). The main component of marjoram essential oils was carvacrol which represented more than 80% in all of the components (Dorman and Deans, 2004). It was explained that the antimicrobial mode of action of the marjoram essential oil is considered to rise mainly from their hydrophobic potential to introduce into the bacterial cell membrane (Mathlouthi et al., 2012). Moreover, marjoram essential oil components can penetrate into the interior of the cell and interact with intracellular sites critical for bacterial activities. More precisely, they are able to inhibit glucosyltransferase enzyme activity, which is responsible in bacteria adhesion to its sites (Cristani et al., 2007; Omara et al., 2014). New research reveals that the marjoram essential oil has antimicrobial activity against *Salmonella* and *E. coli* species that is consistent with the results of this study (Leeja and Thoppil 2007; Omara et al. 2014). The results of antibacterial activity of *Foeniculum vulgare* Mill essential oil showed medium inhibition against *E. coli* and no inhibition against *Salmonella* spp in disc diffusion method but have the less

inhibitory effect on *salmonella* in MIC and MBC method. Similar to these results, Aprotosoiae et al. (2008); Tarek et al. (2014); Abdurahim et al. (2017) reported that *E. coli* are susceptible to *Foeniculum vulgare* Mill. Our results were opposed to those obtained by Bisht et al. (2014) who found that the *Foeniculum vulgare* Mill essential oil showed high antimicrobial activity against *salmonella typhimurium* that this might be difference in bacterial species. According to our study, *Ducrosia anethifolia* essential oil has a moderate inhibitory effect on *E. coli* but has no inhibitory effect on *Salmonella* spp. The main components of the essential oil of leaves and stem of *Ducrosia anethifolia* (α -pinene, myrcene, limonene, terpinolene, and E- β -ocimene) were active against gram positive bacteria that among these components, limonene has the most efficient antimicrobial activity against some gram positive bacteria (Ibrahim, 2001). The previous studies also showed the antibacterial effect of this medicinal plant on various species of Mycobacterium, *Bacillus cereus*, *Bacillus sphericus*, *Bacillus antheracoid*, *Bacillus coagulance*, *Bacillus subtilis* and *Listeria monocytogenes* ATCC 1297 (Habibi et al, 2017; Stavri et al, 2003). So far, no research has been done on the antimicrobial effects of *Ducrosia anethifolia* essential oil on *Salmonella* spp and *E. coli* up to this date. Many studies have reported the antibacterial effects of different *Pulicaria* genus but no study has yet to report about the antibacterial activities of the *Pulicaria gnaphalodes* species against *salmonella* spp and *E. coli* spp. However, it has been reported that the oils and extracts of the different *Pulicaria* species had antibacterial activity against *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Shigella boydii* (Habibi et al, 2018). It has further been reported that the MIC values of *P. gnaphalodes* against *Salmonella typhimurium* and *Staphylococcus aureus* were 0.2 and 0.1 v/v, respectively (Gandomi et al, 2015). The results of this study has shown that *Pulicaria gnaphalodes* essential oil has a moderate inhibitory effect on *E. coli* but has no inhibitory effect on *Salmonella* spp. Khani and Asghari (2012) reported that the most common components of *Pulicaria gnaphalodes* collected from central mountain of Iran include 65% monoterpenes, with α -pinene (34%) and 1,8-cineole (12%) as main compounds, and β -pinen (0.6%), *alloaromadendrene* (0.4%) and *trans-verbenol* (0.2%) as minor compounds were identified in the oil of this plant. Among of these components, the most antibacterial effect has been related to phenolic compound (Nabil Qaid et al., 2014). Nickavar et al. (2002) reported that the gram

positive bacterial strains were more sensitive than the gram negative ones. Nabil Qaid et al. (2014) reported that *E. coli* are more susceptible than *salmonella* spp with the effect of *Pulicaria Inuloides* species that is similar to the results of this study (Nabil Qaid et al. 2014).

CONCLUSION

Pulicaria gnaphalodes L, *Ducrosia anethifolia* L, *Carum copticum* Benth L, *Foeniculum vulgare* Mill and *Majorana hortensis* Minch essential oils have the most inhibitory effect against *E. coli* but there has been less inhibitory effect on *Salmonella* Spp. Avian colibacillosis and salmonellosis are considered to be the avian colibacillosis and salmonellosis are considered to be the major bacterial disease problems in the poultry industry world-wide. In conclusion, *Carum copticum* Benth essential oil contains potential antimicrobial components that may help to prevent and treat some of the poultry diseases associated with *E. coli* and *salmonella* spp.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Author`s contributions

Habibi and Ghahtan were involved in the collection of data, statistical analysis and drafting of the manuscript. Ghahtan and Moramezi read and approved the final manuscript.

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