Tackling experimental colisepticaemia in broiler chickens using phytobiotic essential oils and antibiotic alone or in combination

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Summary

This study was designed to compare the efficacy of a phytobiotic containing a mixture of essential oils of *Oreganum aetheroleum* and an antibiotic containing ciprofloxacin as an active principle for the treatment of experimental *Escherichia coli* (*E. coli*) infection in broiler chickens. Two-hundred-day-old broiler chickens were divided into 5 equal groups. The 1st group was neither challenged nor treated, but groups 2, 3, 4 and 5 were challenged with *E. coli*. The 2nd group was challenged with *E. coli* only, while the 3rd, 4th and 5th group were treated with phytobiotic, ciprofloxacin and phytobiotic and ciprofloxacin combinations, respectively. Results confirmed significant ($P<0.05$) improvement of productive performance parameters, reduction in signs, mortalities, post mortem lesions and bacterial re-isolation, enhancement in cell mediated and humoral immune responses, reduction in levels of liver and kidney function tests and increase in the total protein and globulin levels in challenged chickens treated with either essential oils or ciprofloxacin compared to challenged non treated chickens. Moreover, best significant ($P<0.05$) results in all measured parameters were detected in the group treated with the combination in comparison with those exposed to single treatments. In conclusion, a mixture of essential oils of *Oreganum aetheroleum* is more effective compared with ciprofloxacin in the treatment of *E. coli* in broiler chickens. However, a combined treatment of both could be a superior treatment.

Key words: Poultry, Colibacillosis, Phytobiotics, Ciprofloxacin, Treatment

Introduction

Colibacillosis is a disease in poultry caused by *Escherichia coli* (*E. coli*), especially serotype O78 that induces an acute fatal septicaemia or sub-acute infections (Chansiripornchai and Sasipreeyajan, 2002). Ciprofloxacin is a broad-spectrum, 2nd generation of the fluoroquinolones group (Medders *et al*., 1998). Unfortunately, frequent use of this group may result in the prevalence of fluoroquinolone-resistant *E. coli* in poultry ecosystems, consequently playing a role in the treatment of human *E. coli* infections (Kolář *et al*., 2005). Alternative strategies are therefore being studied including the use of natural additives (Nouzarian *et al*., 2011). Using phytogenic or herbal plants containing essential oils in poultry farms has developed with successful results (Hashemi and Davoodi, 2010). Among these, phytobiotics are species of *Origanum* genus, aromatic plants of the Lamiaceae family. Some studies have demonstrated potent broad spectrum antimicrobial properties of volatile oils of *Origanum vulgare* species (Saeed and Tariq, 2009).

For this reason, the current study was designed to compare the effect of a phytobiotic product containing a mixture of essential oils of *Oreganum aetheroleum* and an antibiotic with an active principle ciprofloxacin for the treatment of experimental infections with *E. coli* in broiler chickens.

Materials and Methods

The used treatments

A commercial phytobiotic natural water additive supplement based on oregano (Orego-stim®) produced by the Meriden Animal Health Company, UK was tested. The main active ingredient of this product is *Oreganum aetheroleum*, produced from the plant *Origanum vulgare*, as it contains many phenolic substances such as carvacrol (81.89%) and thymol (2.12%) as well as monoterpene hydrocarbons γ-terpinene (5.1%) and p-cymene (3.76%). It was used with a dose of 0.3 ml/liter of drinking water for 5 consecutive days. Ciprofloxacin (CIPRO 20%®) is a synthetic fluoroquinolone anti-microbial produced by ArabcoMed Company. Each gram contains 200 mg ciprofloxacin. It was given as 5 mg/kg body weight/day (1 gm/4 liter of drinking water) for 5 consecutive days.

The bacterial culture

An Avian field strain of *E. coli* serotype (O78) was obtained from the Animal Health Research Institute, Dokki, Giza, Egypt. The inoculum was prepared as recommended by Fernandez *et al*. (2002). Each bird in the challenged groups was inoculated intramuscularly with 0.5 ml of the nutrient broth culture containing 10^8 CFU *E. coli* O78/ml.

Antibiotic sensitivity test

In vitro antibiotic sensitivity tests using (Oxoid) discs were adopted according to standard disc diffusion techniques (Prasad *et al*., 1997).

Experimental design

Two hundred twenty one-day-old Hubbard breed of broiler chicks were obtained from Cairo Poultry Company. All chicks were kept in clean, disinfected houses and fed on a balanced commercial ration without antimicrobial agents or feed additives. A vaccination schedule was adopted for the birds. Just before the challenge, twenty chicks were selected randomly and sacrificed to ensure absence of *E. coli* infection. At 14 days old, 200 birds were divided into 5 equal groups, 40 each; the 1st group was not challenged and left as a blank negative control. The 2nd group, kept as positive control, was challenged but not treated. The 3rd and 4th groups were challenged and treated with the phytobiotic and ciprofloxacin, respectively at the recommended doses and durations of the manufacturers. The 5th group was challenged and treated with both phytobiotic and ciprofloxacin. The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).

Evaluation parameters

Productive performance

Average body weight (ABW), feed conversion rate (FCR) and European Production Efficiency Factor (EPEF) of the chickens in each group were determined weekly according to Sainsbury (1984).

Morbidity and mortality rates

The birds in the challenged group were observed daily after the challenge for any symptoms and deaths. Dead birds were examined for specific *E. coli* lesions.

Bacteriological investigation

For bacterial re-isolation, lungs and livers were collected from 10 sacrificed birds in each group at the 1st, 2nd, 3rd and 4th weeks after the challenge. Re-isolation was done as recommended by Sambrook *et al*. (1989).

Immune response

To test for cell mediated immunity, phagocytic activity and index of peripheral blood monocytes were determined using *Candida albicans* at 3 and 5 weeks of age (Chu and Dietert, 1989).

For humral immunity, at the age of 3 weeks, all groups were intramuscularly inoculated with 0.5 ml/bird of a 5% suspension of washed sheep red blood cells (SRBC). The haemagglutinating inhibiting (HI) antibody titers were thereafter determined at 4, 5 and 6 weeks of age (Wegmann and Smithies, 1966).

Serum biochemical metabolites

At 6 weeks of age, the levels of serum aspertate aminotransferase (AST) and alanine aminotransferase (ALT) (liver function tests) were determined together with uric acid and creatinine (kidney function tests) according to Santurio *et al*. (1999) and Sachan *et al*. (2002). Serum samples were also analyzed for total protein and albumin where globulin concentration was detected by subtracting the albumin concentration of the proteins, according to which the albumin to globulin ratio was then calculated (Doumas, 1971).

Statistical analysis

Data were presented as means ± SDs for numerical variables and as percentages for qualitative variables. A one way ANOVA was run to assess the statistical significance of the mean differences between the groups. A Bonferroni Post hoc test was used when the ANOVA was significant. Pearson Chi-square tests were run to show significance relationships between categorical variables. The significance level was set at $P \leq 0.05$. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS©) version 16.0

Results

In vitro antibiotic sensitivity patterns showed that the *E. coli* challenged strain was highly sensitive to ciprofloxacin compared with enrofloxacin, norfloxacin oxytetracycline and neomycin.

Table 1 shows the results of the productive performances (ABW, FCR and EPEF) of all groups from the first to the 6th week. Significant ($P<0.05$) improvements were observed in overall productive parameters in the negative control birds and other challenged treated groups when compared with control positive birds during the whole experimental period. The best significant ($P<0.05$) performance parameters were demonstrated in the group treated concomitantly with oregano essential oils and ciprofloxacin as compared to those treated with each compound separately.

Symptoms appeared 2 days post challenge. Depression, ruffling, anorexia thirst, nasal and ocular discharge, coughing and rals were observed. Severest symptoms (90% morbidity rate) were seen in the positive control challenged group; however, the non challenged ones showed no signs. The severity of symptoms decreased 10 days after being treated with oregano essential oils and ciprofloxacin separately, whereas the response of chickens to the combined treatment with oregano essential oils and ciprofloxacin was more pronounced as the symptoms continued to improve more rapidly post-treatment. Few affected birds showed mild symptoms of respiratory distress 5 days after the end of the treatment.

No deaths occured in the non challenged group; however, challenged non-treated chickens showed a cumulative mortality rate of 40% at the 2nd day post challenge. This percentage decreased in the treated chickens to reach 6, 5 and 3%, respectively in groups 3, 4 and 5. Mortality reduced by the 5th day of the combined treatment with essential oils and ciprofloxacin, and completely disappeared by day 7.

Lesions of dead birds were serous to fibrinous pericarditis, perihepatitis and airsacculitis associated with tracheitis and pneumonia. The mildest (serous) lesions were recorded in challenged groups treated with phytobiotic essential oils and ciprofloxacin alone or in combination as compared to the challenged non-treated groups. Complete absence of the lesions were seen a week after the combined treatment.

Results of bacterial re-isolations are seen in Table 2. This organism was not recovered from challenged chickens treated with ciprofloxacin or phytobiotic essential oils and ciprofloxacin combinations at the 3rd and 4th weeks post challenge. The group treated with phytobiotic essential oils revealed an absence of organism recovery only at week 4 after the challenge. The challenged non-treated group had a higher significant ($P<0.05$) frequency of *E. coli* re-isolation that ranged from 90 to 50% along all the observation period.

The effect of different treatments on cell mediated immune response parameters is illustrated in Table 3. At weeks 3 and 5, challenged positive control birds produced the lowest significant ($P<0.05$) mean values of both phagocytic activity and index compared to other groups. At 3 weeks of age, birds treated with either phytobiotic essential oils or ciprofloxacin revealed significant ($P<0.05$) higher indices compared with challenged non treated chickens. This difference remained significant ($P<0.05$) at the 5th week of age. As compared to the other groups, chickens treated with combined phytobiotic essential oils along with ciprofloxacin showed the best significant ($P<0.05$) mean values.

Data presented in Table 4 reveals the results of (HI) antibody titers against SRBC in different groups. Compared to others, chickens inoculated with *E. coli* had significant ($P<0.05$) decreases in antibody titers at the 1st, 2nd and 3rd weeks post SRBC inoculation. Antibody titers were significantly ($P<0.05$) higher in broilers challenged with *E. coli* and treated with phytobiotic essential oils and ciprofloxacin combined and these titers were significantly ($P<0.05$) higher than those treated with phytobiotic essential oils or ciprofloxacin alone during all sample collection intervals.

Table 1: Effect of oregano essential oils and ciprofloxacin treatments on productive performances in *E. coli* challenged broiler chickens

Group number	Treatment	Average body weight/gm						FCR	EPEF
		Age/week							
		1	2	3	4	5	6		
1	Non challenged + non treated	140.82 ± 3.40^{a}	304.0 ± 5.32^{ab}	598.3 ± 21.8^{a}	940.2 ± 51.2^{a}	1340.2 ± 35.7^{a}	1678 ± 42.30^{a}	1.93	210.03
2	Challenged + non treated	134.98 ± 5.55^{a}	256.4 ± 8.65^{c}	501.1 ± 19.2^{b}	702.91 ± 54.10^{b}	930.12 ± 32.4^{c}	1250.1 ± 14.30^{c}	2.40	145.21
3	Challenged + oregano essential oils	135.74 ± 6.18^{a}	298.1 ± 7.55^{ab}	563.41 ± 23.86^{a}	876.45 ± 71.80^{a}	1119.6 ± 67.2^{b}	1479.1 ± 33.20^{b}	2.00	181.41
4	Challenged + ciprofloxacin	133.96 ± 6.72^{a}	285.9 ± 8.40^{b}	556.31 ± 1.53^{a}	824.99 ± 0.89^{ab}	1089.51 ± 3.2^{b}	1401.9 ± 34.51^{b}	2.2	179.56
5	Challenged + oregano essential oils + ciprofloxacin	140.24 ± 2.37^{a}	307.9 ± 5.30^{a}	584.9 ± 20.11^{a}	906.90 ± 32.09^{a}	1198.0 ± 68.2^{b}	1596.4 ± 39.44^{a}	1.90	205.44

FCR= Feed conversion ratio, EPEF= European production efficiency factor. Means with different letters ($^{a, b, c}$) within the same column are significantly different at $P\leq0.05$

Table 2: Effect of oregano essential oils and ciprofloxacin treatments on *E. coli* re-isolation rate in *E. coli* challenged broiler chickens

Group number	Treatment	Number of examined chickens	Weeks after challenge			
			1st	2nd	3rd	4th
1	Non challenged + non treated	10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
2	Challenged + non treated	10	9/10 (90%)	7/10 (70%)	5/10 (50%)	5/10 (50%)
3	Challenged + oregano essential oils	10	3/10 (30%)	2/10 (20%)	2/10 (20%)	0/10 (0%)
4	Challenged + ciprofloxacin	10	3/10 (30%)	1/10 (10%)	0/10 (0%)	0/10 (0%)
5	Challenged + oregano essential oils + ciprofloxacin	10	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
P-value			<0.001	<0.001	0.003	0.001

Table 3: Effect of oregano essential oils and ciprofloxacin treatments on cell mediated immunity (phagocytic activity and index) in *E. coli* challenged broiler chickens

Group number	Treatment	3 weeks of age		5 weeks of age	
		Phagocytic activity	Phagocytic index	Phagocytic activity	Phagocytic index
1	Non challenged + non treated	54.11 ± 2.83^{bc}	0.5167 ± 1.21^{a}	56.69 ± 3.78^{c}	0.5293 ± 0.22^{b}
2	Challenged + non treated	40.62 ± 1.86^{d}	0.2001 ± 0.12^{a}	48.11 ± 3.20^{d}	0.3010 ± 0.10^{b}
3	Challenged + oregano essential oils	61.31 ± 4.72^{b}	0.5322 ± 0.10^{a}	65.17 ± 2.79^{b}	0.5460 ± 0.10^{b}
4	Challenged + ciprofloxacin	49.78 ± 5.60^{cd}	0.4140 ± 0.31^{a}	51.97 ± 0.22^{cd}	0.4789 ± 0.10^{b}
5	Challenged + oregano essential oils + ciprofloxacin	72.03 ± 0.29^{a}	0.7450 ± 0.02^{a}	75.99 ± 0.89^{a}	0.9889 ± 0.18^{a}

Means with different letters ($^{a, b, c, d}$) within the same column are significantly different at $P\leq0.05$

Table 4: Effect of oregano essential oils and ciprofloxacin treatments on humoral immunity (HI) titers in *E. coli* challenged broiler chickens

Group number	Treatment	Weeks after inoculation of sheep red blood cells		
		1st	2nd	3rd
1	Non challenged + non treated	5.5±0.31[a]	6.1±0.23[a]	5.0±0.21[a]
2	Challenged + non treated	2.91±0.11[c]	3.83±0.30[b]	3.22±0.19[b]
3	Challenged + oregano essential oils	4.90±0.36[ab]	5.4±0.28[a]	5.1±0.36[a]
4	Challenged + ciprofloxacin	4.1±0.40[b]	5.2±0.37[a]	4.9±0.36[a]
5	Challenged + oregano essential oils + ciprofloxacin	5.36±0.56[a]	5.78±0.40[a]	5.1±0.46[a]

Means with different letters ([a, b, c]) are significantly different at P≤0.05

Table 5: Effect of oregano essential oils and ciprofloxacin treatments on liver and kidney functions in *E. coli* challenged broiler chickens

Group number	Treatment	Liver function tests		Kidney function tests	
		AST (IU/L)	ALT (IU/L)	Uric acid (mg/dL)	Creatinine (mg/dL)
1	Non challenged + non treated	201.7±5.83[c]	61.9±4.62[d]	6.92±0.46[d]	0.42±0.99[a]
2	Challenged + non treated	259.4±3.27[a]	90.3±1.25[a]	15.11±0.23[a]	0.60±0.123[a]
3	Challenged + oregano essential oils	230.3±21.31[abc]	77.1±1.23[bc]	10.99±0.20[b]	0.39±0.100[a]
4	Challenged + ciprofloxacin	236.5±8.25[ab]	82.3±1.52[b]	11.53±0.81[b]	0.50±0.130[a]
5	Challenged + oregano essential oils + ciprofloxacin	220.1±9.31[bc]	71.2±2.90[c]	9.45±0.41[c]	0.43±0.121[a]

AST= Aspertate aminotransferase, ALT= Alanine aminotransferase. Means with different letters ([a, b, c, d]) are significantly different at P≤0.05

Table 6: Effect of oregano essential oils and ciprofloxacin treatments on albumin and globulin levels in *E. coli* challenged broiler chickens

Group number	Treatment	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A/G Ratio
1	Non challenged + non treated	2.90±0.23[b]	1.41±0.02[c]	1.49±0.05[c]	0.946±0.03[ab]
2	Challenged + non treated	3.92±0.084[a]	1.97±0.05[a]	1.95±0.04[b]	1.011±0.03[a]
3	Challenged + oregano essential oils	3.85±0.086[a]	1.81±0.042[b]	2.04±0.05[ab]	0.887±0.02[b]
4	Challenged + ciprofloxacin	3.72±0.109[a]	1.80±0.043[b]	1.92±0.08[b]	0.937±0.04[ab]
5	Challenged + oregano essential oils + ciprofloxacin	4.07±0.100[a]	1.90±0.041[ab]	2.17±0.07[a]	0.875±0.02[b]

A/G= Albumin/Globulin. Means with different letters ([a, b, c]) are significantly different at P≤0.05

The effects of different treatments on serum biochemical metabolites parameters are shown in Tables 5 and 6. Challenged non treated chickens demonstrated a significant (P<0.05) increase in the activity of liver enzymes (AST and ALT) and kidney parameters (uric acid and creatinine) (Table 5). On the other hand, for challenged-treated broiler chickens, these parameters decreased significantly (P<0.05) toward the values recorded for negative control birds. Concurrent treatment with phytobiotic essential oils and ciprofloxacin displayed the lowest significant (P<0.05) levels of parameters compared with the separate treatment.

As shown in Table 6, treatment with either phytobiotic essential oils or ciprofloxacin significantly (P<0.05) reduced serum albumin concentration; however, application of the combined treatment resulted in a marked elevation of serum total protein and globulin levels compared to other treatments (P<0.05). The albumin to globulin ratio was lower in non challenged or treated birds than challenged non treated birds. On the other hand, this ratio showed the lowest values in birds treated with phytobiotic essential oils, ciprofloxacin and their combinations.

Discussion

The results of the *in vitro* antibiotic sensitivity pattern agree with those of Blanco *et al*. (1997) who showed that the avian *E. coli* strain was highly sensitive to ciprofloxacin *in vitro*.

The mechanism of phytobiotic essential oils for improving the productive performance of broilers might be caused by improvements in feed conversion ratios and more efficient feed utilization (Mocar *et al*., 2010), which promote better sedimentation of muscle proteins (Zheng *et al*., 2009), stimulation of appetite, digestive and absorption enzymes (Christaki *et al*., 2011) or the stimulating effect on *Lactobacillus* proliferation (Roofchaee *et al*., 2011). Contrary results were obtained by Ocak *et al*. (2008) and Karimi *et al*. (2010) who reported that the performance of growing broilers was not affected by the use of any oregano-based supplement. This discrepancy could probably be due to the ingredient composition and dosage of the used phytogenic compounds (Yang *et al*., 2009). The development of performance parameters in ciprofloxacin treated birds could be attributed to the bactericidal effect of the drug on *E. coli* and the resulting improvement in general health conditions (Brown, 1996).

This work showed that essential oils succeeded in the elimination of the challenged *E. coli* organism. Similar findings were obtained by Bendahou *et al*. (2008), Ouwehand *et al*. (2010) and Rahimi *et al*. (2011) who found that essential oils extracted from the plant

Origanum vulgare had a broad spectrum of antimicrobial activities against avian pathogens either *in vitro* or *in vivo*. High concentrations of essential oils lead to cell membrane lysis and denaturation of cytoplasmic proteins (Helander *et al*., 1998), increase bacterial membrane permeability causing leakage of protons and potassium ions, decrease the pH gradient across the cytoplasm membrane, collapse the membrane potential, inhibit ATP synthesis and consequently induce cells death (Ultee *et al*., 2002). Reduction of the *E. coli* re-isolation in the ciprofloxacin treated group was due to the inhibition of DNA gyrase and Topoisomerase II enzymes needed for the transcription and replication of bacterial DNA (Brown, 1996).

In this study, results of cell mediated and humoral immune responses demonstrated that using phytobiotic essential oils, together with ciprofloxacin, induced an immune potentiating effect. The effect of oregano essential oils on the stimulation of immune responses in chickens is somewhat rare, but thymol and carvacrol have been shown to have potent antioxidant properties which elevate the chicks' immune responses (Gabor *et al*., 2010; Feizi and Nazeri, 2011). Other species of plants contain steroidal saponins that affect human cytokine production, macrophage activation and lymphocyte activity (Tan and Vanitha, 2004). In addition, *Origanum vulgaris* was found to modulate ovine neutrophils immune functions (Farinacci *et al*., 2008). Thyme essential oils were also shown to significantly inhibite total mRNA IL-1B expression in mice colons (Juhas *et al*., 2008). Moreover, essential oils were found to be associated with an increased proportion of swine CD4+, CD8+ and double positive T cells in peripheral blood and mesenteric lymph nodes (Walter and Bilkei, 2004) and thymol was found to enhance total IgA and IgM serum levels, exhibiting local anti-inflammatory properties indicated by a reduction in TNF-mRNA in the swines' stomach (Trevisi *et al*., 2007). Contrasting results were obtained by Toghyani *et al*. (2010), Abdulkarimi (2011) and Mansoub and Myandoab (2011), who noticed that dietary treatments of broiler chickens with thymol did not induce any significant effect on HI antibody humoral immune responses to SRBC and ND viruses. Although our results showed that the ciprofloxacin treatment stimulated either cellular or humoral immune responses, Punniamurthy and Porchezhian (2007) demonstrated that ciprofloxacin had an immunosuppression effect, as it induced a significant reduction of HI antibody titers against SRBC, and did not produce any significant effect against La Sota antigen.

It has been previously suggested that essential oils of oregano have biological antioxidant properties that enhance liver and kidney functions (Hernandez *et al*., 2004). The high antioxidant activity of thymol is due to the presence of phenolic OH groups which act as hydrogen donors to the peroxy radicals produced during the first step of lipid oxidation, thus delaying the formation of hydroxy peroxide (Farag *et al*., 1989). Besides, El-Boushy *et al*. (2006) found out that ciprofloxacin had no hepato or nephro-toxicity in *E. coli* infected broilers. Nevertheless, it is important to mention that plants and their essential oils could be used as hepato and nephro-tonics to counteract the side effects of a number of chemotherapeutic agents (Sylvestre *et al*., 2006).

As compared to the challenged control, the albumin to globulin ratio was the lowest in the treated groups which is a good indicator of high globulin levels and their subsequent immunostimulation.

It could be concluded from the present work that to treat *E. coli* in broiler chickens, a mixture of essential oils of *Oreganum aetheroleum* is more effective than ciprofloxacin. However, a combined treatment of both could be considered as a more effective treatment. For more comprehensive findings, further research in this area is suggested.

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