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EFFECTS OF REPLACING ANTIBIOTIC GROWTH PROMOTERS (AGPS) WITH BOTANICAL EXTRACTS AND OILS IN FEED OF LAYING HENS ON PRODUCTION, PERFORMANCE AND SOME MICROBIAL COUNTS IN FECES

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ABSTRACT

Botanical extracts and essential oils are considered important replacement additives for the antibiotic growth promoters in animal feeds. The aim of the present study was to investigate the effect of botanical extracts and essential oils on production performance of laying hens and microbial count viz; total bacterial count, *E. coli* and *salmonella* presence in feces. One hundred and twelve, 40 weeks old, white Novagin laying hens (1480±50 grams average live body weight each) were randomly assigned to seven groups equally (n = 16). Each treatment group was replicated four times with four birds per cage unit. Experimental diets were prepared by adding polyphenolic extracts of black tea (C), black cumin seed (D), fenugreek seed (E) as well as seed oils from black cumin (F) and fenugreek (G) in the negative control (B; treatment with no antibiotic or antioxidant added in the diet) and compared with positive control **(A)** carrying antibiotic lincomysin (4.4%) 120mg/kg of feed, antioxidant seldox (BHA, BHT, ethoxiquine and citric acid) 120mg/kg of feed, acetic acid (99.5%) 0.15mL/kg of feed. After completion of the five weeks trial, weekly egg production percentage of all treatments were significantly higher than negative control (B) ($P<0.05$), however treatments A, C, D, E, F and G had non-significant differences among one another ($P>0.05$). The presence of *Salmonella* and *E. coli* in feces of the birds on experimental treatments on different days of trial was insignificantly variable but total bacterial count decreases with increasing age except negative control. These results demonstrate that the plant extracts/oils have positive affect on the production performance and microbial health of the laying hens and thus can be used instead of commercial antibiotics.

Key words: layer feed, botanical extracts, *Salmonella*, *E. coli.*

INTRODUCTION

The research on incorporation of plant extracts in poultry feed is gaining importance due to their positive impact on health and performance of birds as well as improved egg quality and yield (Lokaewmanee *et al.,* 2009; An *et al.,* 2010; Mahmoud *et al.,* 2010; Hu *et al.,* 2011; Duru, 2013; Zhao *et al.,* 2013; Boka *et al., 2*014). It has also been established that the use of antibiotics as growth promoter in chicken feed results in tissue residues (Botsoglou and Fletouris, 2001). Therefore, use of natural plant and herb resources in order to replace antibiotic growth promoters (AGPs) to achieve drug free poultry products is increasing (Madrid *et al.,* 2003). Tea leaves contain 36% polyphenolic contents, 19% proteins and and less than 0.1% carotenoids and other volatile contents (Akram *et al.,* 2012). Among polyphenolic contents of tea, flavanols e.g. catechin, epicatechin, epicatechingallate and epigallocatechingallate perform prime functional role (Chaturvedula and Prakash, 2011). The beneficial influence of tea, its extract and powder on animal production and quality has been reported (Yosef *et al.,* 2012). Black cumin seeds possess antioxidant activity (Guler *et al.,* 2006; Guler *et al.,* 2007; Aydin *et al., 2*008). These possess alkaloids, fixed and volatile oils and a variety of functional compounds. It has been reported that the broiler and layers performance and egg quality improved by offering black cumin seed in feed (Akhtar *et al.,* 2003; Guler *et al.,* 2006; Abu-Dieyeh and Abu-Darwish, 2008; Aydin *et al.,* 2008). It is noted that feeding 2 or 3% black cumin enhanced the egg production, weight and shell quality (Aydin *et al., 2*008). Black cumin seeds (*Nigella sativa*) can be used to replace antibiotics because of its pharmacological properties related to production performance and health of poultry. Similarly, fenugreek showed antiviral, antioxidant, antifungal and anti-carcinogenic effects (Abaza, 2007). The use of fenugreek in hen feed has been investigated for its antioxidant characteristics (Safaa, 2007; Abaza, 2007; Criste *et al., 2*013). Incorporation of 1% germinated fenugreek seeds resulted in increased egg production with no adverse effects on egg quality (Hassan *et al., 2*004). The supplementation of layers feed with 0.5% fenugreek seeds improved the egg production gain by 2.23% as compared to that of the control group and significantly decreased feed intake was observed as well (Abaza, 2007). Thus plant derived feed additives such as essential oils or plant extracts could be considered as alternative to the antibiotic growth promoters (Ayasan, 2011). Dash *et al*. (2011) showed

that the botanical extracts of *Coriandrumsativum*L. and *Trigonellafoenum*L. contain effective antimicrobial agents. Sultan *et al*. (2009) reported that black cumin seed has nutraceutical activity to eliminate many disease threats because of its known phytochemistry especially because of the presence of thymoquinone and tocopherols. Nair *et al*. (2005) studied antibacterial effect of black cumin seeds on *Listeria monocytogen*. Similarly Bolukbapi *et al*. (2009) showed that ileal *E. coli* growth was checked by *Nigella sativa* oil in laying hens.

The objective of this study was to evaluate antimicrobial effects of dietary supplemented extracts of black tea, black cumin seeds and fenugreek. Oil fractions of black cumin seed and fenugreek seeds were also evaluated for performance and antimicrobial potency in layers. Various microbial safety indicators like Fecal coliform, *Salmonella* and total bacterial count were investigated to evaluate the comprehensive impact of plant fractions application in poultry diets.

MATERIALS AND METHODS

Extract Preparation from black cumin seed, fenugreek, and black tea: The *black cumin seed, fenugreek, and black tea* samples were extracted using method of Kim and Lee (2002) with slight modifications. Dried samples (200 g) of each plant material were soaked in flask for 24 hours with 1000 mL of 80% (v/v) methanol, filtered with filter paper. The residues collected on filter paper were extracted once more time with 1000 mL 80% (v/v) methanol using sonication. The extracts obtained in result of these two extractions were combined and evaporated in rotary evaporator under vacuum at 40°C until the volume was reduced to 300 ml. The volumes were made 400 ml with water for each sample to standardize crude liquid contents and stored in refrigerator before further use.

Extraction of Oil and Polyphenols from Oil seeds: One kg of each oil seed i.e. black cumin seeds and fenugreek was ground to fine material using an electric grinder (Braun, Frankfurt Germany). In order to avoid losses of heat sensitive antioxidants, the oil was extracted with chloroform-methanol (1:1) mixture through method described by Bligh and Dyer (1959) with the intention of recovering polyphenols.

Experimental trial on layers: Experiment was conducted during August 2013 at Animal House, PCSIR Laboratories Complex, Karachi. One hundred and twelve, 40 weeks old, white Novagin laying hens (1480±50 grams average live body weight each) were randomly assigned to seven groups equally (n = 16). Each treatment was replicated four times with four birds per cage (50x46x46 cm).

Composition of the experimental diets is presented in Table 1 and table 2. The diets were isocaloric and isonitrogenous. Various experimental diets were prepared with variations given as below:

A: Positive control (antibiotic lincomysin 4.4% 120 mg/kg of feed, antioxidant seldox (BHA, BHT, ethoxiquine and citric acid) 120mg/kg of feed,
B: Negative control (N.C), no antibiotic or antioxidant,
C: N.C + black tea poly phenolic extract 1mL/kg of feed,
D: N.C + black cumin seed poly phenolic extract 1mL/kg of feed,
E: N.C + fenugreek seed poly phenolic extract 1mL/kg of feed,
F: N.C + black cumin seed oil 1mL/kg of feed,
G: N.C + fenugreek seed oil 1mL/kg of feed.

Experiment started from 40th week and ended at 45th week. During the experiment, hens were fed 110 grams/day feed in such a way that four birds were offered 440 grams feed in a day and they ate it completely within few hours and water offered them at *ad libitum*.

Eggs production was recorded daily from each cage. Weight of yolk, albumen, shell, and Hough unit values were measured after every 10th day using 8 eggs from each dietary treatment. At each 10th day of experiment, feces samples were taken from each replicate cage in order to determine the presence of *Salmonella, E. coli* and total bacterial count.

Media and Reagents: Both commercially available and laboratory made media were used for isolation, identification and characterization of *E. coli and Salmonella spp.* The media used for bacteriological analysis were Soybean Casein Digest medium, Lactose broth, Peptone water Eosin-Methylene-Blue agar (EMB, Merck) and MacConkey (MC) agar (Merck), Deoxycholate Citrate Agar (DCA) and Propylene Glycol Deoxycholate Agar (PGDA) and Triple Sugar Iron Agar (TSIA).

Bacteriological Analysis

Determination of bacterial load: The total viable cell counts were performed by the spread-plate method. Ten g of fecal sample was dissolved in 100 mL of Fluid Soybean- Casein Digest Medium. Tenfold serial dilutionwere made in 0.1% (v/v) peptone solution. One mL of sample from 7th dilution was pipetted on to sterile petri dishes and 20 mL of Soybean Casein Digest Agar Medium was promptly added to each dish. Then contents were solidified at room temperature. Inverted petri dishes were incubated at 37°C for 24 hours and so identification of bacteria were done based on their morphological, staining, cultural, hemolytic and biochemical properties described by Chessbrough (1985).

Escherichia coli determination: Ten g of fecal sample was taken in clean sterile test tube. A hundred ml volume was made with fluid lactose medium. Tubes were kept for

incubation at 35°C for 24 hrs. After incubation, a portion from test tubes was streaked on MCA medium plates using the inoculating loop and petri plates were let for 24 hrs. incubation at 35°C.

After incubation time brick red colonies were observed, for further identification inoculated these suspected colonies on Eosin-Methylene Blue Agar Medium. After incubation time a characteristic metallic sheen under reflected light and blue –black appearance under transmitted light was observed and presence of *E. coli* was confirmed (Collee *et al.*, 1989).

Salmonella analysis: The isolation of Salmonella from poultry droppings was carried out according to the method described by Barrell (1982) and Collee *et al*. (1989). Approximately 10 g of poultry droppings was introduced into 500ml flasks containing 200ml of selenite F broth (Merck). The inoculated flasks were rotated for proper mixing and incubated for 24h at 42°C. Sub-cultures were thereafter made on to plates of DCA and PGDA media (Morinigo *et al.*, 1986). The plates were incubated at 37°C for 24h. Salmonella typical colonies on the plates (pale or colorless with or without black centers on DCA and a bright red color on PGDA) were cultured onto TSIA medium by first streaking the surface of slant and then stabbing the wire well beneath the surface, incubated at 37°C for 24h. Suspected Salmonella colonies were hydrogen sulfide positive on TSIA tubes having alkaline red slants and acid (yellow) butt.

The data for hens' performance parameters as well as total microbial count was analyzed by using Minitab R 17.1.10 by applying Two Way ANOVA and Tukey's test (Steel *et al.*, 1997).

RESULTS

In the present study, the treatment B (negative control) showed significantly lower egg production than all other treatments ($P<0.05$), as shown in (Table 3). However among all other treatments, non-significant variation ($P>0.05$) was recorded for egg production (Table 3). The comparisons for weekly egg weight, yolk weight and albumin weight (Table 4), revealed that there is no significant difference among treatments ($P>0.05$), whereas shell weight and Hough unit value (Table 4) increased with onset of age. The *salmonella and E. coli* were fond present in all the feces samples collected from birds under different supplemental treatments while the bacterial count decreased significantly ($P<0.05$) from 10th day towards 30th day of age except negative control B (Table 4).

Table1. Formulation of layer feed offered during trial (40-45 weeks).

Ingredients	Percentage
Corn	39.345
Rice broken	5.4
Rice polishing	10
Wheat bran	2
Soybean meal 45%	10
Canola meal	2.5
Guar meal	3
Sunflower meal 25%	10
Fish meal 50%	7
Lime stone /marble	7.8
Salt (NaCl)	0.110
Sodium bi carbonate	0.05
Lysine HCL	0.03
Dl methionine	0.12
Premix (vitamin/mineral)	0.5
Molasses	2.1
Natuzyme (cocktail enzyme)	0.035
Quantum blue (phytase enzyme)	0.01

Table 2. Calculated analysis of prepared feed for layers

Parameter	Results
Crude Protein	16%
ME	2700 kcal/kg
Ash	12.16%
Moisture	11.4%
Calcium	3.51%
Phosphorous (available)	0.47%
Sodium	0.16%
Chloride	0.17%
Digestible lysine	0.76%
Digestible methionine	0.49%
Diges. M+C	0.69%
Diges. Threonine	0.55%
Diges. Tryptophan	0.14%
Diges. Arginine	0.99%

Table 3. Comparison for weekly egg production among different treatments

Treatments	Weekly egg production (%age)				
	Week 1	Week 2	Week 3	Week 4	Mean*
A	87.5	87.5	77.67	85.41	84.52A
B	75	75	68.75	68.75	71.87B
C	81	80.46	79.46	79.68	80.15AB
D	80.35	64.06	59.82	64.84	80.15AB
E	80.35	73.44	73	80	76.69AB
F	87	83	80	84	83.5AB
G	75	76	79.46	84.34	76.69AB

A:Positive control (antibiotic lincomysin 4.4% 120 mg/kg of feed, antioxidant seldox (BHA, BHT, ethoxiquine and citric acid) 120mg/kg of feed, **B**: Negative control (N.C), no antibiotic or antioxidant, **C**:N.C + black tea poly phenolic extract 1mL/kg of feed, **D**: N.C + black cumin seed poly phenolic extract 1mL/kg of feed, **E**: N.C + fenugreek seed poly phenolic extract 1mL/kg of feed, **F**: N.C + black cumin seed oil 1mL/kg of feed, **G**: N.C + fenugreek seed oil 1mL/kg of feed.

*Any two means not sharing a letter in the column differ significantly at P<0.05.

Table 4. Results for egg physical quality characteristics in birds under different treatments

Treatments	Egg weight (gm)			Shell weight (gm)			Yolk weight (gm)			Albumen weight (gm)			Hough unit Value		
	Day 10	Day 20	Day 30	Day 10	Day 20	Day 30	Day 10	Day 20	Day 30	Day 10	Day 20	Day 30	Day 10	Day 20	Day 30
A	56.62	62.58	56.5	7.11	8.62	8.17	16.1	16.22	15.31	35.1	37.59	35.76	100	101	100
B	54.85	57.75	60.23	6.75	7.79	7.68	16.03	16.37	15.68	31.75	33.5	37.01	89.6	101	102
C	54.3	55.04	56.49	6.86	7.54	7.34	15.8	16.11	15.22	29.82	31.61	33.79	89.4	103	108
D	59.47	52.28	57.34	7.88	7.4	8.34	15.58	14.03	15.17	35.21	30.6	33.77	95.4	102	107
E	58.45	55.7	58.09	7.44	8.1	7.81	14.25	15.42	16.08	35.43	32.12	33.97	104	104	109
F	56.15	52.28	57.68	6.68	6.94	8.12	15.72	13.63	16.22	35.32	31.6	32.56	102	104	102
G	56.79	56.19	57.17	7.32	7.97	7.44	15.5	16.66	14.59	33.57	31.32	34.92	103	106	105
Mean*	57.64 A	56.66 A	55.97 A	7.14 B	7.76 A	7.84 A	15.56 A	15.49 A	15.46 A	34.54 A	33.74 A	32.62 A	97.58 B	103.0 AB	105.0 A

A:Positive control (antibiotic lincomysin 4.4% 120 mg/kg of feed, antioxidant seldox (BHA, BHT, ethoxiquine and citric acid) 120mg/kg of feed, **B**: Negative control (N.C), no antibiotic or antioxidant, **C**:N.C + black tea poly phenolic extract 1mL/kg of feed, **D**: N.C + black cumin seed poly phenolic extract 1mL/kg of feed, **E**: N.C + fenugreek seed poly phenolic extract 1mL/kg of feed, **F**: N.C + black cumin seed oil 1mL/kg of feed, **G**: N.C + fenugreek seed oil 1mL/kg of feed.

*Any two means not sharing a letter in the last row differ significantly at P<0.05.

Table 5. Results for total bacterial count (cfu/gm) determination for different treatments

Treatments	Total bacterial count (cfu/gm)		
	Day 10	Day 20	Day 30
A	1000	1000	800
B	1300	1300	1300
C	1500	1000	1000
D	1300	1300	1200
E	1300	1200	1000
F	1500	1300	1200
G	1600	1400	1000
Mean*	1300 A	1171.43 AB	1028.57 A

A:Positive control (antibiotic lincomysin 4.4% 120 mg/kg of feed, antioxidant seldox (BHA, BHT, ethoxiquine and citric acid) 120mg/kg of feed, **B**: Negative control (N.C), no antibiotic or antioxidant, **C**:N.C + black tea poly phenolic extract 1mL/kg of feed, **D**: N.C + black cumin seed poly phenolic extract 1mL/kg of feed, **E**: N.C + fenugreek seed poly phenolic extract 1mL/kg of feed, **F**: N.C + black cumin seed oil 1mL/kg of feed, **G**: N.C + fenugreek seed oil 1mL/kg of feed.

*Any two means not sharing a letter in the last row differ significantly at P<0.05.

DISCUSSION

All diets with plant extracts and vegetable oils i.e. C, D, E, F, G and positive control A showed non-significant effect with respect to egg production however negative control had showed significantly lowered egg production. The results also showed the reduction in pathogens counts at par to the antibiotic and organic acid bearing treatment of control diet. These results are in contrast to Kojima and Yoshida (2008) who reported the reduction in egg production when layers were fed with 5% and 10% green tea powder. However two layer groups which were offered feeds with 1% green tea powder or control showed no reduction in production of egg. On the other hand, reduction in bacterial count in feces of treatments under the influence of botanical extracts and oils has been reported. The control of micro flora of gut by these extracts can lead to reduction in competition for nutrients and decrease in growth inhibitory metabolites (Catala-Gregori *et al., 2*008). Dash *et al*. (2011) showed that the botanical extracts of *Coriandrumsativum* L. and *Trigonellafoenum* L. contain effective antimicrobial agents. Sultan *et al*. (2009) reported that black cumin seed has nutraceutical activity to eliminate many disease threats because of its known phytochemistry especially because of the presence of thymoquinone and tocopherols etc. Similar study using black cumin etc. as source of antibacterial activity was conducted by Nair *et al*. (2005). Bolukbapi *et al*. (2009) showed that ileal *E. coli* growth was checked by *Nigella sativa* oil in laying hens. However Erener *et al*. (2010) showed non significant effect on coliform count in broiler chicks with black cumin seed or its extract in diet. Bourgou *et al*. (2010) explained that the antibacterial activity of black cumin seeds is due to the thymoquinone substances such as terpenes. Bakkali *et al*. (2007) observed that the antibacterial effects of black cumin may be linked with the efficacy of its essential oil, which can disturb mitochondrial membranes and cellular function of bacteria and eukaryotic cells and result in cell death. Lavinia *et al*. (2009) also showed the beneficial effects of essential oils of plants, on the intestinal absorption by cells and immunity level responses. Wenk (2002) showed that polyphenolic extracts of plants improved birds' performance.

Egg weight, shell thickness, albumen weight, yolk weight and Hough unit value differed insignificantly in test diets with positive control diet as well as negative control diet during present study. It shows that the quality of egg remains constant despite the botanical extracts treatment. However egg weight and Hough unit value elevated with increase in age which is observed as a natural trend. Azeke and Ekpo (2008) presented that addition of 1% and 2% black tea into laying hen feed had no effect on egg weight. Similarly, Uuganbayar *et al.* (2005) showed no effect on egg weight by feeding 1.0% or 1.5% green tea powder. However, reduction in egg weight by feeding 0.5% green tea was reported by Xu *et al.* (2009). Biswas *et al*. (2000) reported that by feeding 0.6% green tea egg weight was negatively affected. Whereas feed conversion ratio was improved by feeding 0.5%, 1.5%, and 2.5% black tea powder (Xu *et al., 2*009). Aydin *et al.* (2008) showed that by black cumin at 2 or 3% level could significantly affect egg production, shell strength and egg weight. However these treatments had no effect on albumin quality. In comparison to these results, Bolukbapi *et al*. (2009) showed that feeding hens by 3 mg/kg *Nigella sativa* oil decreased Hough units. The improvements in albumin quality may be because of alkaloid and saponin contents and also the antioxidant effects of black cumin such as carvacrol and thymoquinone (Denli *et al., 2*004). Many studies have shown that the bioactive material of medicinal plants have strong power to keep safe magnum and uterus cells and also increase the level of albumin in laying hens (Denli *et al., 2*004; Nadia *et al., 2*008). Aydin *et al*. (2008) reported that black cumin had little effect on egg shape, yolk index, and egg shell weight; whereas inclusion of 2% and 3% black cumin can enhance egg shell thickness and shell strength. Similarly it was observed that egg shell thickness and yolk index improved by feeding at least 0.5% black cumin (Akhtar *et al., 2*003). Bolukbapi *et al.* (2009) proposed that feeding of *Nigella sativa* oil up to 3 ml/kg of hen's diet had insignificant effect on egg weight. Aydin *et al*. (2008) and Yalcin *et al*. (2009) suggested that dietary black cumin seed feeding at the rate of 10, 15 and 30 g/kg respectively, increased the egg weight. The inclusion of black cumin seed at the levels of 0.05–1.0% (El-Sheikh *et al.,* 1998) and 1.5% (Akhtar *et al., 2*003) of diet as well as *Nigella sativa* extracts at the rate of 1 g/ kg of diet (Denli *et al., 2*004) increased egg production. However in contrast to these findings, a number of studies showed ineffectual results of black cumin seed and oil use in feed on production of eggs (Aydin *et al., 2*006; Bolukbapi *et al., 2*009; Yalcin *et al., 2*009). In contrast, dietary black cumin inclusion (10 and 30 g/kg) has been reported showing insignificant variation in egg production percentage (El-Bagir *et al., 2*006). Yalcin *et al*. (2009) reported that the dominant fatty acid of plant glycolipid fractions from black cumin seeds is linoleic acid (C 18:2 n-6), and oleic acid (C 18:1 n-9). These both fatty acids have prime role in egg size. Al-Beitawi and El-Ghousein (2008) noticed that feeding low level (1.5%) of black cumin could increase the efficiency of broiler chicks.

Polyphenolic contents may reduce pathogenic bacteria and their toxins from feed and elementary canal, thus consequently may increase the enzyme activity. In the present study, no incidence of animal diseases was reported in all treatments. The low *Salmonella* and *E coli* count in the feces in all treatments groups remained at par ($P>0.05$) to the positive control group. It justifies that

botanical extracts can control harmful bacteria like *Salmonella* and *E. coli* at similar level to antibacterial drug i.e. lincomysin The concept of application of plant extracts to control micro-organisms correlates with Madrid *et al*. (2003) who demonstrated that the thyme extract reduced the *Coliform* count. Similar studies have reported that essential oils derived from spices and herbs could be successfully used as growth promoters. These increased feed intake under the influence of their aromatic characteristics in chicken which increases digestive enzymes and the improved utilization of digestive products through enhanced liver function (Langhout, 2000; Hertrampf, 2001). Present study explains that botanical extracts can improve health of birds while having insignificant effect on egg production. On the other hand total bacterial concentration reduced with the increasing age of layer except negative control, thus *Salmonella* and *E coli* counts may also be reduced. It has also been reported that plant extracts e.g. carvacol, cinnamaldhyde and capsaicin reduced the total *E. coli* count in intestine of broilers chickens (Jamroz *et al., 2*003). Sarica *et al.* (2005) found that the broilers fed with thyme (0.1%) had significantly lower *E. coli* count than the control diet in the small intestine. Tucker (2002) reported that the supplementation of a mixed herbal product containing garlic, anise, cinnamon, rosemary and thyme to commercial pig diets significantly inhibited the number of *E. coli* in the digestive tract. The count of *Salmonella* and *E. coli* in feces of the birds on experimental treatments on different days of trial, was insignificantly variable (P>0.05). These results demonstrate that the plant extracts/oils have shown potential in controlling microbial growth and thus can be used instead of commercial antibiotics.

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