IMMUNOLOGY, HEALTH, AND DISEASE

Peganum harmala Can Minimize *Escherichia coli* Infection in Poultry, but Long-Term Feeding May Induce Side Effects

N. Arshad,*† C. Neubauer,* S. Hasnain,† and M. Hess*[1]

*Department for Farm Animals and Herd Management, Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria; and †Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

ABSTRACT *Peganum harmala* seed extracts have been frequently reported to possess antibacterial potential through in vitro studies, but in vivo studies have acquired less attention. The present study was therefore designed to investigate its efficacy on the course of colibacillosis and effects of long-term feeding on selected parameters of general health in chickens. Two experiments were conducted in this regard. Experiment 1 (a pilot study) was performed to determine the dose of a field strain of *Escherichia coli* (O1:K1) required to induce clinical symptoms in 4- and 15-d-old specific-pathogen-free chickens. A successful induction of colibacillosis, in terms of clinical signs, mortality, and pathological lesions in addition to reisolation of the pathogen was observed by inoculating 4- and 15-d-old chicks with 4.3 $\log_{10}$ and 6.4 $\log_{10}$ cfu of *E. coli*, respectively, by intraperitoneal injection. Using these doses experiment 2 (main study) consisting of a single experiment with 3 parts was performed. Parts A and B generated the information regarding efficacy of the extract against infection in 4- and 15-d-old chickens applying different treatment schemes, whereas the effects of continuous feeding of the extract were assessed in part C. Whereas no protective effect of the extract could be recorded in young chickens, significant differences ($P < 0.05$) with regard to BW, clinical score, gross lesion score, and total granulocyte counts were observed in 15-d-old birds. Bacterial recovery per gram of tissue and reisolation frequency were lower in treated birds. The continuous feeding of the extract for 6 wk resulted in an augmentation in relative liver weight and depletion in alkaline phosphatase, protein, albumin, and globulin. It can be concluded that the crude extract of *Peganum harmala* possesses limited antimicrobial activity against *E. coli* in vivo and long-term continuous feeding may induce undesired effects. Furthermore, the study underlines the value of in vivo experiments and the diverse picture that herbal products, in this case *Peganum harmala*, may deliver by testing them against specific pathogens.

Key words: *Peganum harmala*, antimicrobial, in vivo, colibacillosis, poultry

2008 Poultry Science 87:240–249
doi:10.3382/ps.2007-00341

INTRODUCTION

Bacterial infections in poultry remain of great importance world-wide in terms of economic effects and public health. Antibiotic therapy and prophylaxis are widely used, keeping in mind the associated problems of developing resistant pathogens (Barrow, 1997; Saenz et al., 2001; Altekruse et al., 2002). As a consequence of existing reports about multidrug resistance, the fear of the general loss of effective antimicrobials is increasing (FDA, 2001). This problem has led to an intensified research for alternative treatments. Plant products are considered as one of the active research areas in this context. Medicinal plants are being explored with the intention of finding new antimicrobial agents, especially against drug-resistant bacteria. Some of these plants have been reported to possess broad-spectrum antimicrobial activity against resistant bacterial strains (Fukai et al., 2004; Dickson et al., 2006; Kuźma et al., 2007). In vitro results of many plant extracts are being reported with great frequency from different laboratories (Cowan, 1999; Ríos and Recio, 2005). However, fewer data are available regarding in vivo testing of these extracts (Lamchouri et al., 2002; Guo et al., 2004; Stipkovits et al., 2004; Lamien et al., 2005). Only few studies are available on the exploitation of plant extracts as antimicrobial agents using in vivo infection models (Stipkovits et al., 2004; Bandyopadhyay et al., 2005; Prakash, 2006).

Peganum harmala, although considered as a noxious weed, has some beneficial effects. The smoke of its seeds is traditionally used as a disinfectant (Shahverdi et al., 2005). Its seed extracts are known to contain β-carbolin alkaloids, anthroquinons, and a small quantity of flavo-

Received August 17, 2007.
Accepted October 22, 2007.
[1]Corresponding author: michael.hess@vu-wien.ac.at

noid glycosides (Sharaf et al., 1997; Prashanth and John, 1999). There are reports that alkaloids in *Peganum harmala* seed are mainly responsible for different pharmacological activities including antibacterial effects in addition to vasorelaxant, antihemosporidian, anticancer, antinociceptive, antitumor or antineoplastic, and antiprotozoal effects (Adaay et al., 1989; Fan et al., 1997; Lamchouri et al., 1999; Lala et al., 2004; Monsef et al., 2004; Chen et al., 2005; Berrougui et al., 2006). However, in vivo studies focusing on their responses against bacterial infections in poultry have not yet been documented. Moreover, therapeutic doses (1 g/kg of BW) of this plant have been reported to be nontoxic in a rodent model (Lamchouri et al., 2002). This result would justify further studies in other animals.

In the in vitro studies, ethanol and methanol extracts of *Peganum harmala* seeds have been found to inhibit the growth of several avian pathogenic *Escherichia coli* resistant to a lot of antibiotics (unpublished data). The major objective of present study was therefore to test in vivo the activity of *Peganum harmala* as an antibacterial agent in an *Escherichia coli* (induced) infection model. Two experiments, experiment 1 (a pilot study) and experiment 2 (a main study), were performed in specific-pathogen-free (**SPF**) chickens. The pilot study was conducted to define the infection dosage of a field strain of *E. coli* (O1:K1). The main study was divided into 3 parts; parts A and B addressed the question of efficacy of the seed extract against experimental colibacillosis in 4- and 15-d-old chickens using different application schemes for the extract, whereas part C was designed to determine the effects of long-term feeding on selected parameters of general health of the birds. All experimental procedures complied with the regulations of the Commission for Animal Trials in Austria with respect to animal experimentation and care of animals under license no. 68-205/01-42 BrGT/2006.

MATERIALS AND METHODS

Bacteria and Culture Conditions

The *E. coli* strain used in the present studies was isolated from diseased chicken showing typical pathological signs and pathological lesions of colibacillosis. It was characterized as avian pathogenic *E. coli* serotype O1:K1 on the basis of the standard slide agglutination method (Orskov et al., 1977). The challenge strain was resistant to cefacetril, erythromycin, lincomycin, metronidazol, penicilline G, spiramycin, spectinomycin, tetracycline, and tylosin as determined by microdilution method, using commercially available antibiotic coated plates (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany). The pure culture was stored at –80°C and refreshed on blood agar plates (BioMerieux, Vienna, Austria) before use.

Inoculum Preparation

The *E. coli* strain was revived on McConkey agar plates (BioMerieux, Vienna, Austria) by culturing overnight at 37°C. A single colony of fresh culture was transferred in Lauria Britanica broth and cultured for 18 to 24 h at 37°C. The bacteria were harvested by centrifugation at 3,380 × *g* for 15 min. The inoculum was prepared by washing this pellet twice with PBS, and the optical density was adjusted at 1 ± 0.002 at 600 nm by SmartSpec plus photometer (BioRed Inc. Laboratories, Vienna, Austria), corresponding to approximately 10^8 cfu/mL. Tenfold dilutions of this suspension were used to inoculate different groups of chicks. The actual viable counts in each dose were determined by plate count method, for which different dilutions of the inocula were plated on MacConkey agar plates. Counts were conducted after 24 h following incubation at 37°C.

Plant Extract Preparation

Seeds of *Peganum harmala* were soaked in 96% ethanol (10 g/100 mL). Extraction was performed in a sonicator (Transsonic 470/H, Elma, Singen, Germany) at room temperature for 2 h; the resulting extract was filtered, concentrated in a rotary evaporator, dried in a freeze drier, and stored at 4°C until use. The extract was dissolved in PBS (200 mg/mL). To facilitate solubility, it was kept at 37°C overnight with continuous shaking. The exact concentration of this solution was determined by subtracting the amount of undissolved extract. Later on, it was filter sterilized through 0.2-μm filter (Sarstedt, Nuembrecht, Germany) and administered to the birds at different concentrations as described below.

Animals and Housing Conditions

A total of 144 specific pathogen-free (**SPF**) chickens (Valo; Lohmann-Tierzucht, Cuxhaven, Germany) hatched at the Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine, Vienna, Austria, were used in the study. The chickens were distributed randomly and kept under controlled conditions in sterilized isolation units (Montair Andersen B.V. HM 1500, Sevenum, the Netherlands; size: 1.2 m^2) with the airflow of 30 to 32 m^3/h. The temperature was adjusted at 37°C during the first week of life and later on reduced gradually (2°C per week) to 20°C by the age of 6 wk. Light period was kept at 12 h throughout the trial. Feed and water were provided ad libitum.

Experiment 1 (Pilot Study)

Establishing an Inoculation Model. A total of 36 chickens were divided into 5 groups (I to V). Group I (n = 12) was kept as negative control, and groups II to V (n = 6) were inoculated with different selected doses of *E. coli*. Groups II and III were inoculated at d 4 of life with 3.3 $\log_{10}$ and 4.3 $\log_{10}$ cfu, whereas groups IV and V received inocula at d 15 of life containing 5.4 $\log_{10}$ and 6.4 $\log_{10}$ cfu, respectively. Clinical signs and mortality were recorded daily. Six days postinoculation (**dpi**), all surviving birds were killed and the experiment was

Table 1. Design of experiment 2 (main study)

		Part A			Part B			Part C	
		I	II	III	IV	V	VI	VII	VIII
Treatment	Age (d)	n = 9	n = 15	n = 15	n = 9	n = 15	n = 15	n = 15	n = 15
Infection	4	−	+	+	−	−	−	−	−
Plant extract[1]	4	−	−	+	−	−	+	+	−
Necropsy	10	+	+	+	−	−	−	−	−
Infection	15				−	+	+	+	−
Plant extract[2]	15				−	−	−	+	−
SRBC[3]	15				−	−	−	+	+
Necropsy	21				+	+	+	−	−
Sampling	25 and 40							+	+
SRBC[3]	25							+	+
PHA[4]	41							+	+
Necropsy	42							+	+

[1]Plant extract was given continuously from d 4 onwards to groups III, VI, and VII at the concentration of 75 ± 15 mg/kg of BW/d.

[2]The dose of extract was increased to 150 ± 25 mg/kg of BW.

[3]Sheep red blood cells (SRBC) were given by intraperitoneal injection.

[4]Phytohemagglutinin (PHA) was injected subcutaneously.

terminated. Dead birds as well as killed birds were investigated for specific lesions indicative for colibacillosis. Liver, heart, spleen, and lungs were removed aseptically for bacteriological examination, and samples were streaked out directly on McConkey agar plates.

Experiment 2 (Main Study)

In the main study, 108 birds were divided in 8 groups (I to VIII). To investigate specific objectives, the birds from groups I to III were used for part A, those from groups IV to VI for part B, and the 2 remaining groups VII and VIII in part C of the study (Table 1).

Part A. Efficacy of the Extract in 4-D-Old Chickens. A total of 39 chicks were divided in 3 groups (I to III). Group I (n = 9) served as negative control, and birds received 0.5 mL of PBS by intraperitoneal injection. Birds kept in group II (n = 15) and group III (n = 15) were infected with 4.3 $\log_{10}$ cfu in a volume of 0.5 mL of PBS. For treatment, birds in group III received the plant extract (75 ± 15 mg/kg of BW) by crop gavage using a syringe with feeding needle once daily starting at day of inoculation until 6 dpi. Following the same method of application, birds in groups I and II received the same quantity of PBS. Clinical signs and mortality were recorded daily. Surviving birds were euthanized at 6 dpi, and blood samples were collected for serological studies from all birds. Necropsy was performed on dead and euthanized birds. Gross lesions were scored according to a defined scheme (see below), and liver, heart, lung, and spleen were removed aseptically for microbiological examination. Blood samples were analyzed for glutamate oxaloacetate transaminase (**GOT**), lactate dehydrogenase (**LDH**), alkaline phosphatase (**ALP**), protein, albumin, and globulin.

Part B. Efficacy of the Extract in 15-D-Old Chickens. The experimental design was essentially the same as in part A, except that the feeding of the plant extract began at d 4 of age (75 ± 15 mg/kg of BW) and animals were inoculated at d 15 of life by injecting 0.5 mL of PBS containing 6.9 $\log_{10}$ cfu of the pathogen. Moreover, the dose of extract was increased to 150 ± 25 mg/kg of BW following day of inoculation. The same parameters were investigated as mentioned above for birds of part A. In addition, total erythrocyte and granulocyte counts together with packed cell volume and bacterial recovery per gram of tissue (liver, heart, lung, and spleen) were recorded from the birds killed at 6 dpi.

Part C. Effect of Long-Term Feeding of the Extract on Clinical Observations, Blood Biochemistry and Immunological Responses of SPF Chickens. A total of 30 birds were equally divided in 2 groups (VII and VIII), of which birds in 1 group (VII) received the plant extract once daily from d 4 until termination of the study when the birds were 6 wk old. The extract administered from d 4 until d 14 of life was 75 ± 15 mg/kg of BW. Later on, the dose was increased up to 150 ± 25 mg/kg of BW. The control group (VIII) was treated similarly with PBS. The birds were investigated daily for any adverse clinical signs. The effects of extract feeding on the immune status of the birds were evaluated by taking into account the humoral and cell-mediated immune responses. For humoral immune response, heparinized sheep blood was washed twice in PBS (pH 7.2), and 1 mL of 1% suspension of erythrocyte was injected by the intraperitoneal route in birds of both groups at d 15 and 25 of life. Blood samples were collected 10 and 15 d following first and second exposure to sheep red blood cells (**SRBC**), respectively. Serum was separated and stored in aliquots at –20°C until further use. Moreover, cell mediated immune response was recorded using a phytohemagglutinin (**PHA**) mediated skin thickness test after 40 d of plant extract treatment. Animals were killed at d 42 of age. Blood samples were collected and used for clinical biochemistry including determination of GOT, LDH, ALP, protein, albumin, and globulin and the im-

munological responses of birds (antibody titer to SRBC). Postmortem examination included recording of the whole BW and determination of the relative weight of liver, spleen, and bursa. Gross morphological lesions on liver, heart, lung, and spleen were recorded, and bacteriological investigation of these organs was performed.

Scoring Scheme and Laboratory Procedures

Clinical Scores. The health status of the birds was scored from 0 to 4 on the basis of following criteria: 0 = animal active with no clinical symptoms; 1 = slightly weak, dropping wings, diarrhea; 2 = depressed with swollen crop; 3 = weak with ruffled feathers, reluctant to walk, and apathy; and 4 = animal unable to move or stand, eyes closed, and intense breathing. The health status was scored daily from day of inoculation to the day of termination of experiment.

Gross Pathological Lesion Score. Tissue lesions from liver and heart were scored according to Mellata et al. (2003). The scoring scale for different organs was as follows: (i) Liver: 0 = normal; 1 = slight amounts of fibrinous exudate; 2 = marked perihepatitis. (ii) Heart and pericardium: 0 = normal; 1 = vascularization, opacity, cloudy fluid in the pericardial cavity; 2 = acute pericarditis. (iii) Lung: 0 = normal; 1 = edema; 2 = edema and hyperaemia; 3 = edema, hyperemia and fibrin in air sacs. (iv) Spleen: 0 = normal; 1 = swollen 2 = fibrinated bedding.

Bacteriological Examinations of Tissues (Qualitative Examination). The presence of the bacterial strain used for infection was determined qualitatively by streaking the samples from liver, lung, heart, and spleen directly on McConkey agar plates. The plates were incubated overnight at 37°C for 24 h and observed for the presence of *E. coli*.

Bacterial Recovery (Quantitative Examination). Liver, heart, lung, and spleen (100 to 200 mg) were homogenized in 1 to 2 mL of PBS and 100 μL of serial dilutions of the homogenate were spread on McConkey agar plates for bacterial quantification. Moreover, 1 mL of the homogenate was incubated overnight in LB broth to investigate the presence of *E. coli* in the tissue samples given above.

Hematology and Clinical Biochemistry. Hematological investigations were performed on heparinized blood samples taken from birds during euthanization. Erythrocyte counts and PCV were measured following Swarup et al. (1986), whereas granulocytes were counted using eosinophil unopette method (Campbell, 1995). For erythrocyte counts the blood was diluted (1:200) in Natt and Herrick (1952) solution, and for granulocyte count it was diluted (1:20) in unopette solution, which stains only heterophils and eosinophils, the number of cells were counted in 9-mm area in a Neubauer chamber.

For clinical biochemistry, plasma was separated by centrifuging blood at 3,380 × *g* for 15 min, and GOT, LDH, ALP, total protein, and albumin were measured on automated clinical chemistry analyzer Hitachi 911 (Roche Diagnostics, Mannheim, Germany) with reagent test kits supplied by Roche. Globulin was determined as a difference between total protein and albumin (Varley, 1975).

Humoral Immune Response. Antibodies against SRBC were measured by quantifying total antibody titer in addition to mercaptoethanol sensitive IgM and mercaptoethanol resistant IgG using microagglutination assay (Delhanty and Solomon, 1966). Briefly, 2-fold serial dilutions of serum were prepared in PBS in microtiter plates; later, an equal volume of 1% SRBC in PBS was placed in all wells. Plates were shaken for 1 min and incubated for 1 h at 37°C for total antibody titer. The agglutination titer was expressed as $\log_2$ of the highest dilution of sera giving visible agglutination. For IgG the test was performed exactly in the same manner except that the plasma was incubated with equal volume of 0.2 *M* of 2-mercaptoethanol for 1 h at room temperature before making 2-fold dilutions. The IgM was calculated as a difference of total immunogloubin and IgG titer. Primary antibody titer against SRBC was estimated from the serum samples collected after 10 d of first exposure to SRBC, whereas the secondary antibody titer was estimated from the sera taken at the day of termination of the experiment.

Cell-Mediated Immune Response. The PHA skin test for T-cell-mediated immunity was conducted in 41-d-old chickens following the procedures of Grasman and Scanlon (1995) using a 0.1 mL dose of 1 mg/mL of PHA-P (Sigma, St. Louis, MO) in PBS. Feathers were plucked from both wing webs. One wing was injected with PHA, whereas the other received a placebo injection of PBS alone. The thickness of each wing web was measured to the nearest 0.05 mm immediately before and 24 ± 3 h after the injections, using vernier caliper with the precision of 0.01 mm. A stimulation index was calculated as the change in the thickness of the PHA-injected wing web minus the change in thickness of the PBS-injected wing web.

Statistical Analysis

The quantitative data are presented as mean ± SEM and were analyzed using 1-way ANOVA followed by Duncan's multiple range test (DMRT), or Student's *t*-test. The qualitative data were analyzed following χ^2 test of independence. All analyses were performed in SPSS software for window version 14 (SPSS Inc. USA). Probability values ≤ 0.05 were considered statistically significant.

RESULTS

Experiment 1 (Pilot Study)

Establishing an Inoculation Model. Six days postinoculation, the chicken inoculated at d 4 of age with 3.3

Table 2. Pilot study: Effects of different doses of field strain on avian pathogenic *Escherichia coli* on the outcome of colibacillosis

Age (d)	Group[1] (n = 6)	Dose (cfu)	Body weight (g)	Morbidity[2] (%)	Mortality (%)	Reisolation of *E. coli*[3]				Birds positive for infection[4]
						Liver	Lung	Heart	Spleen	
4	I	Uninfected	82.50 ± 3.82	0	0	0	0	0	0	0/6 (0%)
	II	3.3 $\log_{10}$	91.17 ± 4.57	0	0	3	4	2	2	6/6 (100%)
	III	4.3 $\log_{10}$	84.17 ± 5.19	33	33	3	6	2	2	6/6 (100%)
15	IV	Uninfected	250.71 ± 5.82	0	0	0	0	0	0	0/6 (0%)
	V	5.4 $\log_{10}$	181.00 ± 0.98	50	33	4	3	2	3	5/6 (83%)
	VI	6.4 $\log_{10}$	186.67 ± 0.14	100	66	3	6	5	3	6/6 (100%)

[1]Groups I and IV served as negative control, whereas groups II, III, V, and VI were infected intraperitoneally with *E. coli*.

[2]Animals expressing clinical signs.

[3]Number of birds from which the infective strain of *E. coli* was reisolated.

[4]The birds from which *E. coli* was reisolated at least from a single organ were considered as positive for infection. The ratio of positive and infected birds is displayed.

$\log_{10}$ cfu of *E. coli* O1:K1 remained clinically healthy. No significant difference in BW was observed comparing infected vs. control groups. During pathological examination, only 2 out of 6 chickens showed gross lesions in lungs. However, the higher dose, 4.3 $\log_{10}$ cfu, resulted in 33% (2/6) mortality. During necropsy gross lesions were found in several organs in 83% (5/6) birds. From every infected bird (6/6) *E. coli* O1:K1 could be reisolated, at least from 1 organ (Table 2). At both doses the frequency of reisolation was highest in lungs, followed by liver, heart, and spleen.

In 15-d-old chickens inoculated with 5.4 $\log_{10}$ cfu, 3 birds showed adverse clinical signs characterized by apathy, lethargy, and ruffled feathers, and 2 chickens died. The surviving birds were weak and killed at 6 dpi. During necropsy, gross lesions were observed in lungs (edema and hyperaemia), liver (cloudy), and heart (mild fibrin), whereas no lesions were observed in the spleen of the birds. Mortality in birds infected with the higher dose (6.4 $\log_{10}$ cfu) was 66%; 4 of the 6 birds died. All birds had lesions in the lungs characterized by edema, hyperaemia, and airsacculitis. In 4 of the birds, pericarditis was recorded. Three birds showed perihepatitis with involvement of liver and spleen. The reisolation pattern was very much similar to the situation noticed in the birds inoculated at 4 d of age.

Experiment 2 (Main Study)

Part A. Efficacy of the Extract in 4-Day-Old Chickens. The chickens inoculated with 4.4 $\log_{10}$ cfu at the

Table 3. Effects of *Peganum harmala* (seed extract) treatment on some pathological parameters of specific-pathogen-free (SPF) chicken infected with 4.4 $\log_{10}$ cfu of *Escherichia coli* (O1:K1) at the age of 4 d

Parameter[1]	Group		
	Uninfected (n = 9)	Infected (n = 12)	Infected and treated (n = 15)
Mortality	0	3/15	0/15
Body weight	67.22 ± 4.09	63.33 ± 3.51	64.33 ± 3.08
Protein (g/dL)	2.83 ± 0.19	2.66 ± 0.12	2.93 ± 0.14
Albumin (g/dL)	1.30 ± 0.05	1.34 ± 0.07	1.27 ± 0.10
ALP[2] (U/L)	2,473.60 ± 1,235.90	2,918.11 ± 461.83	1,694.50 ± 552.16
GOT[3] (U/L)	191.80 ± 37.98	242.56 ± 20.46	174.44 ± 29.83
LDH[4] (U/L)	661.40 ± 233.13	1,313.00 ± 267.33	919.44 ± 180.85
Reisolation[5]			
Liver	0	7/12	4/15
Lung	0	8/12	4/15
Heart	0	7/12	4/15
Spleen	0	7/12	4/15
Total	0	29/48	16/60
Sum of lesion score			
Liver	0	5	2
Lung	0	18	14
Heart	0	3	2
Spleen	0	2	1

[1]Parameters were recorded at 6 d postinfection.

[2]Alkaline phosphatase.

[3]Glutamate oxaloacetate transaminase.

[4]Lactate dehydrogenase.

[5]Number of samples positive vs. number of samples investigated are displayed.

Table 4. Effects of *Peganum harmala* seed extract on selected parameters in specific-pathogen-free (SPF) chickens infected with 6.9 $\log_{10}$ cfu of *Escherichia coli* (O1:K1) at the age of 15 d

	Group		
Parameter[1]	Uninfected (n = 9)	Infected (n = 7)	Infected and treated (n = 6)
Mortality	0	8/15	9/15
Protein (g/dL)	3.53 ± 0.09	3.46 ± 0.17	3.36 ± 0.29
Albumin (g/dL)	1.99 ± 0.04	1.62 ± 0.08	1.80 ± 0.09
ALP (U/L)	1,352.00 ± 302.08	1,434.14 ± 418.07	1,946.50 ± 536.38
GOT (U/L)	208.66 ± 11.79	232.85 ± 57.21	180.33 ± 7.38
LDH (U/L)	584.00 ± 34.15	679.42 ± 162.01	514.50 ± 41.93
PCV	208.00 ± 11.79	149.00 ± 8.35	177.00 ± 15.28
Bacterial load/g of tissue[2] ($\log_{10}$)			
Liver	0.00 ± 0.00	3.35 ± 0.52	1.70 ± 0.59
Lung	0.00 ± 0.00	4.56 ± 0.55	3.02 ± 0.62
Heart	0.00 ± 0.00	3.97 ± 0.82	2.29 ± 0.50
Spleen	0.00 ± 0.00	3.69 ± 0.81	2.23 ± 0.73
Total	0.00 ± 0.00	3.89 ± 0.33*	2.31 ± 3.89
Reisolation[3]			
Liver	0/9	4/7	1/6
Lung	0/9	4/7	2/6
Heart	0/9	5/7	3/6
Spleen	0/9	5/7	2/6
Total	0/9	18/28*	8/24

[1]Parameters were recorded at 6 d postinfection.

[2]Bacterial load of infected and infected treated group was compared by Student's *t*-test.

[3]Samples positive vs. investigated are displayed, comparison between infected and infected treated group was performed using χ^2-test.

*$P < 0.05$.

age of 4 d did not give any reportable findings. No marked differences in any pathological or clinical biochemical parameter could be observed in plant treated and nontreated birds despite the fact that the infection was successfully demonstrated by reisolation of the bacteria from different organs combined with lesions observed in the lungs of the birds from both groups. The only differences were a higher mortality (3/15) and reisolation frequency (29/60) in the nontreated group compared with the treated group (0/15 and 16/60, respectively). However, the differences were not statistically significant (Table 3).

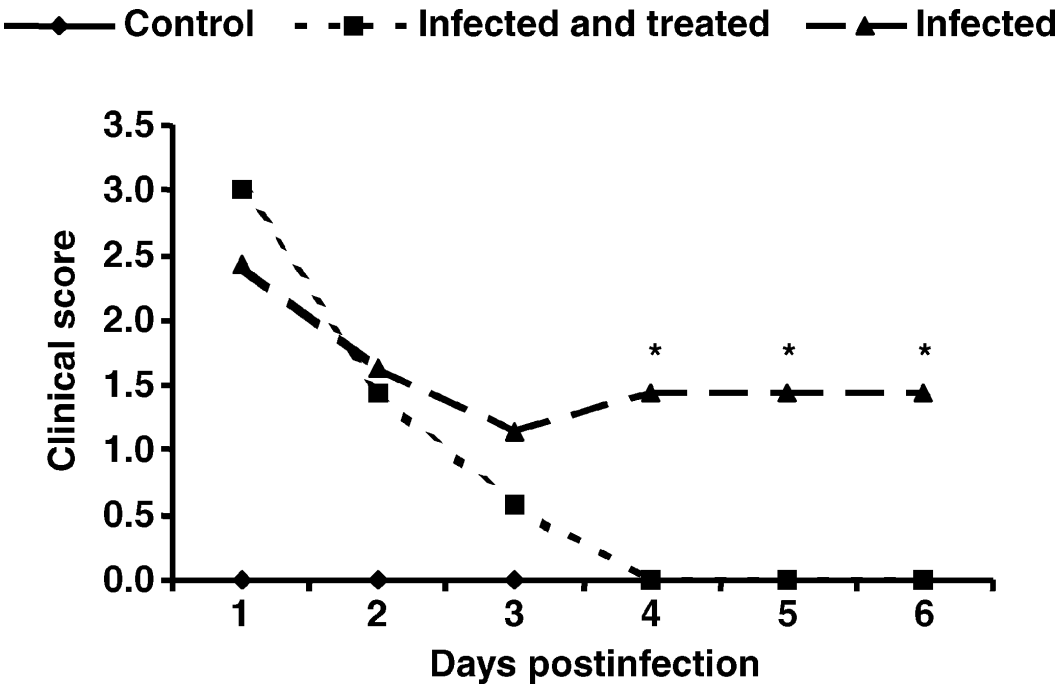

Figure 1. Effects of *Peganum harmala* seed extract on clinical score of SPF chicken infected with 6.9 $\log_{10}$ cfu of *Escherichia coli* (O1:K1) at the age of 15 d. *Indicates significant differences ($P > 0.05$).

Part B. Efficacy of Extract in 15-D-Old Chickens. The effects noticed for the treatment with the plant extract in 15-d-old chickens are presented in Table 4, Figure 1, and Figure 2. Mortality in treated (9/15) and nontreated (8/15) birds was similar. However, in surviving chickens the clinical score recorded for the treated group dropped to the normal level after 3 dpi, whereas it remained high in the nontreated group (Figure 1). Bacteria were reisolated from 8 organs out of a total of 24 investigated in treated birds and 18 out of 28 in nontreated birds. The mean bacterial recovery/g of tissue was 2.31 and 3.89 $\log_{10}$ in the treated and nontreated birds, respectively ($P < 0.05$). However, the differences were not significant when comparison was made between individual organs.

Regarding hematological studies, total granulocyte counts were significantly higher in the inoculated group compared with the control and the treated groups (Figure 2) but total erythrocyte counts and packed cell volume remained unaltered. The BW of the treated birds was higher than the nontreated group, even though it was less than the BW in the control group (Figure 2). Moreover, no significant differences could be recorded in the clinical biochemical parameters including GOT, ALP, LDH, total protein, albumin, and globulin.

Part C. Effects of Long-Term Feeding of the Extract in SPF Chickens. No adverse clinical signs could be observed after continuous feeding of the plant extract over a period of 6 wk except slight depletion in BW ($P > 0.05$; Table 5). During necropsy a significant increase in relative liver weight was noticed. Blood biochemical evaluation revealed depletion in some blood parameters including GOT, ALP, total protein, albumin, and globulin was recorded. However, LDH level and immunological parameters including primary and secondary antibody titer to SRBC and responses to PHA remained unaltered.

DISCUSSION

Peganum harmala seed extracts are reported to contain alkaloids, flavonoids, and anthroquinons (Sharaf et al., 1997; Prashanth and John, 1999). The alkaloids in the seed extract have been utilized to control hemosporidian infection in naturally and experimentally infected cattle (Fan et al., 1997; Hu et al., 1997). Until now, the effect of *Peganum harmala* seed extract was only studied in mammalian models, and its therapeutic doses have been reported to be safe for rodents (Lamchouri et al., 2002). Presently no information is available concerning the effect of this plant in poultry. Therefore, the present investigation was set up to determine the antibacterial potential of *Peganum harmala* extract derived from seeds in SPF birds infected with *E. coli* to induce colibacillosis. The first obstacle in this regard was to establish an inoculation model. Pathogenic *E. coli* are known to harbor different sets of genes responsible for pathogenicity (La Ragione and Woodward, 2002; Ewers et al., 2005), and variable outcomes of infection could be associated de-

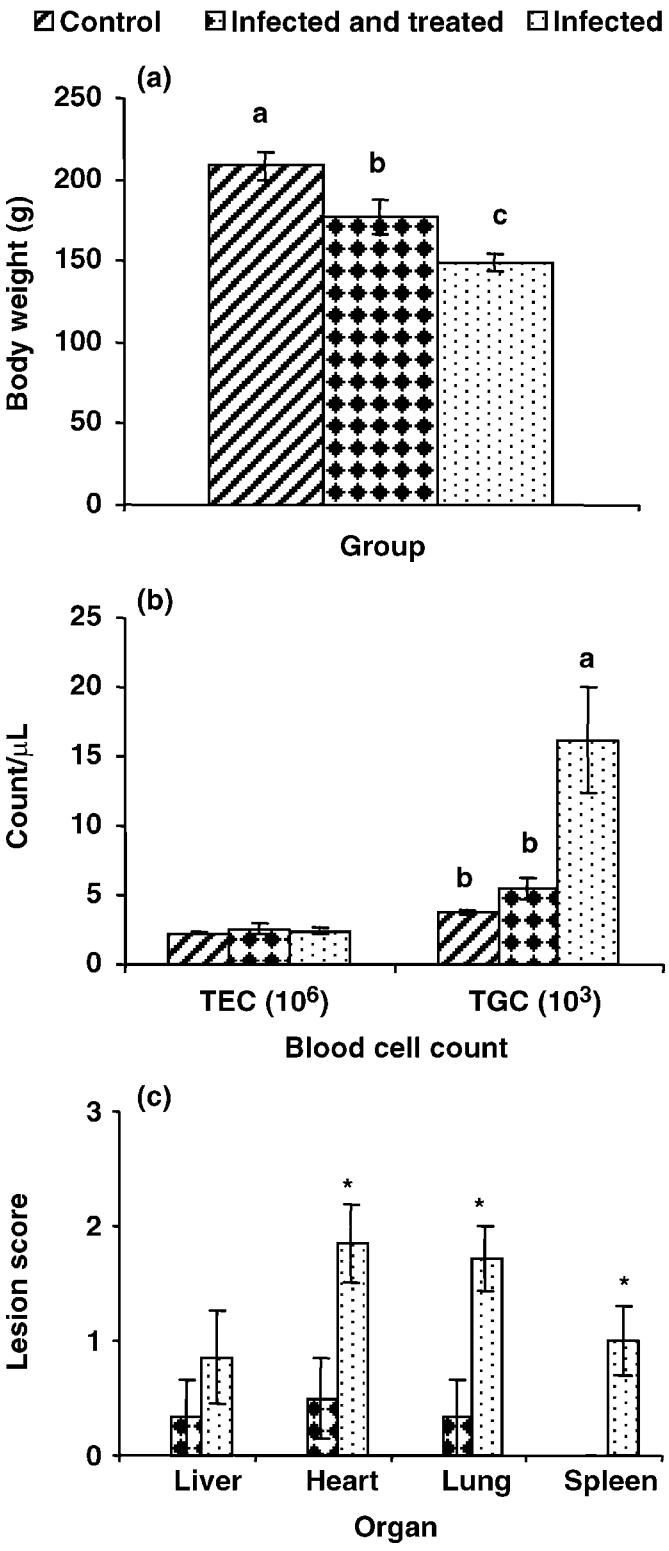

Figure 2. The effects of *Peganum harmala* seed extract on (a) BW, (b) total erythrocyte count (TEC) and total granulocyte counts (TGC), and (c) gross lesion score in SPF chicken infected at 15 d of life with 6.9 $\log_{10}$ cfu of *Escherichia coli* (O1:K1). Observations were recorded 6 d postinfection. Legends for panels a–c are the same. Values are mean ± SEM. In part (a) and (b), bars having different letters are significantly different. *Asterisks in part (c) show significant differences.

Table 5. Effects of continuous feeding (6 wk) of *Peganum harmala* seed extract on clinical observations; blood biochemistry and immunological responses of specific-pathogen-free (SPF) chicken

		Group	
Diagnostic study	Parameter	Control (n = 15)	Treated[1] (n = 15)
	Mortality	0	0
Clinical observation	Clinical score	0	0
	Gross lesion score	0	0
	Body weight	509.0 ± 25.07	481.43 ± 17.10
Relative organ weight (g)	Liver	0.023 ± 0.001	0.029 ± 0.001*
	Bursa	0.005 ± 0.000	0.005 ± 0.000
	Spleen	0.002 ± 0.000	0.002 ± 0.000
Clinical biochemistry	Total serum proteins (g/dL)	3.64 ± 0.09	3.10 ± 0.06*
	Total serum albumin (g/dL)	1.65 ± 0.03	1.48 ± 0.03*
	Globuline (g/dL)	1.99 ± 0.08	1.62 ± 0.04*
	ALP (U/L)	4,649.43 ± 771.53	1,529.36 ± 134.9*
	GOT (U/L)	220.71 ± 4.46	186.79 ± 5.24*
	LDH (U/L)	591.29 ± 28.48	488.00 ± 46.76
Immunological response	PHA[2] (mm)	0.51 ± 0.19	1.19 ± 0.62
	Total Ig[3]	3.00 ± 0.63	3.77 ± 0.54
	IgG[3]	0.00 ± 0.00	0.38 ± 0.27
	IgM[3]	3.00 ± 0.63	3.34 ± 0.61
	Total Ig[4]	3.73 ± 0.37	3.46 ± 0.40
	IgG[4]	2.93 ± 0.25	2.50 ± 0.27
	IgM[4]	2.02 ± 0.58	1.78 ± 0.59

[1]Extract was fed to birds once daily (75 ± 15 mg/kg of BW in the first 2 wk and 150 ± 25 mg/kg of BW until the end of the experiment).

[2]Cell mediated immune response to phytohemagglutinin (PHA).

[3]Primary antibody titer against SRBC (log_2).

[4]Secondary antibody titer against SRBC (log_2).

*$P < 0.05$.

pending on the type of pathogen (Landman and Cornelissen, 2006). No information was available on the *E. coli* strain O1:K1 used in the present investigations, and therefore experiment 1 (a pilot study) was conducted to determine the appropriate dose required to induce colibacillosis. The intraperitoneal administration of 4.3 log_{10} and 6.4 log_{10} cfu of this pathogen resulted in mortality as well as clinical signs and specific gross lesions as described by Barnes et al. (2003) in 4- and 15-d-old chickens, respectively, and the scoring system reported by Mellatta et al. (2003) could be adopted. Consequently, the above-mentioned doses were used in the main study.

To test the antibacterial potential of the extract, 4-d-old SPF birds were treated with a dose of 75 ± 15 mg/kg of BW after inoculation. Compared with the birds of the untreated group, these birds indicated slight improvement in the course of infection. From these observations, it could be surmised that the low dose of the extract might be the underlying factor.

The inoculation of 15-d-old birds with 6.9 log_{10} cfu of pathogen resulted in an acute infection leading to 50 to 55% mortality within the first 2 d. However, the surviving birds of the treated group displayed signs of improvements including normalization of clinical scores, regain in BW, lessening of gross lesion score, normal level of granulocyte counts, and decreased bacterial recovery from different organs. These data are an indication for a possible beneficial role of *Peganum harmala* seed extract against colibacillosis. Considering the application scheme in the present study, it could be either a prophylactic or a therapeutic effect. The clinical biochemical tests (total protein, albumin, ALP, GOT, and LDH) were performed to assess any adverse effect of the extract. Imbalanced levels of these enzymes are used as the indicators of diseases and toxicity (Varley, 1975). In this part of the study, the level of all these parameters remained unaltered, corroborating that the extract is harmless for the birds at this dose level. Overall, these results are in agreement with earlier report about the testing of seed extracts of *Peganum harmala* in animals (Fan et al., 1997; Hu et al., 1997).

The continuous feeding of the extract for 6 wk (experiment 2, part C) presented significant impact on the liver function tests, as determined by reduction in total protein (14.8%), albumin (10.3%), globulin (18.6 %), ALP (67.1%), and GOT (15.4%), whereas relative liver weight increased by 26%. Elevated activities of these parameters usually indicate liver or muscle damage, but no particular significance is associated with low activity profiles in birds (Viveros et al., 2002). Lamien et al. (2005) also reported a significant depletion in the GOT level in chickens after feeding decoctions from galls of *Guiera senegalensis*, another plant known to contain alkaloids. This could be an indication that alkaloids may suppress hepatic activity. The decrease in the level of GOT, ALP, total protein, and albumin may be due to (i) inhibition in their synthesis at the transcriptional or translational level, (ii) inhibition in their secretion from the cells, or (iii) increased loss caused by proteinuria due to renal diseases or malnutrition (Varley, 1975; Zantop, 1997).

Interestingly, no influence on the above-mentioned parameters was noticed when the extract was fed to the birds for only 3 wk (part B, group VI). As the crude extract was used in the present study, it is difficult to comment on the components responsible for the adverse effect noticed after long-term feeding. The seed extracts of *Peganum harmala* contains 4 different beta-carboline alkaloids (harmaline, harmine, harmalol, and peganin) of which harmaline and harmine are the major constituents (Sobhani et al., 2002). Of these, harmine has been reported to possess genotoxic potential, whereas harmaline has been found to be least toxic (Di Giorgio et al., 2004).

The findings of the present investigation as well as the antimicrobial potential reported by Ahmad et al. (1992) and Saify et al. (2005) substantiate further studies to assess the antibacterial effects of different components of the extract. In conclusion, the present study provides evidence that the extract derived from seeds of *Peganum harmala* possess some antibacterial properties in vivo; however, the crude extract could not be utilized as continuous feed additive because it probably interferes with hepatic function in chickens.

ACKNOWLEDGMENTS

The research was partially supported by research grant (20-139, 2005) from Higher Education Commission, Pakistan. Authors are thankful to Chlodwig Franz and Karin Zitterl-Eglseer, Institute of Applied Botany and Pharmacognosy, Department of Public Health in Veterinary Medicine, University of Veterinary Medicine Vienna, Austria, for providing facilities for the preparation of the extract.

REFERENCES

Adaay, M. H., L. J. Rashan, K. D. Sulayman, M. Al-Abaar, and T. Ayoub. 1989. Antimicrobial activity of different extracts from the seeds of *Peganum harmala*. Fitoterapia 60:363–365.

Ahmad, A., K. A. Khan, S. Sultana, S. Bina, B. S. Siddiqui, S. Begum, S. Faizi, and S. Siddiqui. 1992. Study of the in vitro antimicrobial activity of harmine, harmaline and their derivatives. J. Ethnopharmacol. 35:289–294.

Altekruse, S. E., F. Elvinger, K. Y. Lee, L. K. Tollefson, E. W. Pierson, J. Eifert, and N. Sriranganathan. 2002. Antimicrobial susceptibilities of *Escherichia coli* strains from a turkey operation. J. Am. Vet. Med. Assoc. 221:411–416.

Bandyopadhyay, D., T. K. Chatterjee, A. Dasgupta, J. Lourduraja, and S. G. Dastidar. 2005. In vitro and in vivo antimicrobial action of tea: The commonest beverage of Asia. Biol. Pharm. Bull. 28:2125–2127.

Barnes, J. H., J. P. Valliancourt, and W. B. Gross. 2003. Colibacillosis. Pages 631–656 in Diseases of Poultry. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, ed. Iowa State Press, Ames.

Barrow, P. A. 1997. Novel approaches to control of bacterial infections in animals. Acta Vet. Hung. 45:317–329.

Berrougui, H., C. Martin, A. Khalil, M. Hmamouchi, A. Ettaib, E. Marhuenda, and M. D. Herrera. 2006. Vasorelaxant effects of harmine and harmaline extracted from *Peganum harmala* L. seeds in isolated rat aorta. Pharmacol. Res. 54:150–157.

Campbell, T. W. 1995. Avian haematology. Page 10 in Avian Haematology and Cytology 2nd ed. Iowa State University Press, Ames.

Chen, Q., R. Chao, H. Chen, X. Hou, H. Yan, S. Zhou, W. Peng, and A. Xu. 2005. Antitumor and neurotoxic effects of novel harmine derivatives and structure-activity relationship analysis. Int. J. Cancer 114:675–682.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12:564–582.

Delhanty, J. J., and J. B. Solomon. 1966. The nature of antibodies to goat erythrocytes in the developing chicken. Immunology 11:103–113.

Dickson, R. A., P. J. Houghton, P. J. Hylands, and S. Gibbons. 2006. Antimicrobial, resistance-modifying effects, antioxidant and free radical scavenging activities of *Mezoneuron benthamianum* Baill., *Securinega virosa* Roxb. & Wlld. and *Microglossa pyrifolia* Lam. Phytother. Res. 20:41–45.

Di Giorgio, C., F. Delmas, E. Ollivier, R. Elias, G. Balansard, and P. Timon-David. 2004. In vitro activity of the beta-carboline alkaloids harmane, harmine, and harmaline toward parasites of the species *Leishmania infantum*. Exp. Parasitol. 106:67–74.

Ewers, C., T. Janssen, S. Kiessling, H. C. Philipp, and L. H. Wieler. 2005. Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis. 49:269–273.

Fan, B., J. Liang, J. Men, F. Gao, G. Li, S. Zhao, T. Hu, P. Dang, and L. Zhang. 1997. Effect of total alkaloid of *Peganum harmala* L. in the treatment of experimental haemosporidian infections in cattle. Trop. Anim. Health Prod. 29(Suppl. 4):77–83.

FDA (Food and Drug Administration). 2001. Antibiotic resistance from down on the chicken farm. A discussion paper by Linda Bren. WebMD http://www.fda.gov/fdac/features/2001/101_chic.html Accessed May 2007.

Fukai, T., Y. Oku, A. J. Hou, M. Yonekawa, and S. Terada. 2004. Antimicrobial activity of hydrophobic xanthones from *Cudrania cochinchinensis* against *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus*. Chem. Biodivers. 1:1385–1390.

Grasman, K. A., and P. F. Scanlon. 1995. Effects of acute lead ingestion and diet on antibody and T-cell-mediated immunity in Japanese quail. Arch. Environ. Contam. Toxicol. 28:161–167.

Guo, F. C., R. P. Kwakkel, B. A. Williams, H. K. Parmentier, W. K. Li, Z. Q. Yang, and M. W. Verstegen. 2004. Effects of mushroom and herb polysaccharides on cellular and humoral immune responses of *Eimeria tenella*-infected chickens. Poult. Sci. 83:1124–1132.

Hu, T., B. Fan, J. Liang, S. Zhao, P. Dang, F. Gao, and M. Dong. 1997. Observations on the treatment of natural haemosporidia infections by total alkaloid of *Peganum harmala* L. in cattle. Trop. Anim. Health Prod. 29(Suppl. 4):72–76.

Kuźma, Ł., M. Rózalski, E. Walencka, B. Rózalska, and H. Wysokińska. 2007. Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: Salvipisone as a potential antibiofilm agent active against antibiotic resistant staphylococci. Phytomedicine 14:31–35.

Lala, S., S. Pramanick, S. Mukhopadhyay, S. Bandyopadhyay, and M. K. Basu. 2004. Harmine: Evaluation of its antileishmanial properties in various vesicular delivery systems. J. Drug Target. 12:165–175.

Lamchouri, F., A. Settaf, Y. Cherrah, M. El-Hamidi, N. Tligui, B. Lyoussi, and M. Hassar. 2002. Experimental toxicity of *Peganum harmala* seeds. Ann. Pharm. Fr. 60:123–129.

Lamchouri, F., A. Settaf, Y. Cherrah, M. Zemzami, B. Lyoussi, A. Zaid, N. Atif, and M. Hassar. 1999. Antitumour principles from *Peganum harmala* seeds. Therapie 54:753–758.

Lamien, C. E., A. Meda, E. Couacy-Hymann, A. G. Ouedraogo, and O. G. Nacoulma. 2005. The phytochemical composition and in vitro antiviral activity of decoctions from galls of *Guiera senegalensis* J.F. Gmel. (Combretaceae) and their rela-

tive non-toxicity for chickens. Onderstepoort J. Vet. Res. 2:111–118.
Landman, W. J., and R. A. Cornelissen. 2006. Virulence factors of *Escherichia coli*, with emphasis on avian pathogenic isolates. Tijdschr. Diergeneeskd. 131:822–830.
La Ragione, R. M., and M. J. Woodward. 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res. Vet. Sci. 73:27–35.
Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss, III, P. K. Brown, P. Arne, A. Bree, C. Desautels, and J. M. Fairbrother. 2003. Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect. Immun. 71:536–540.
Monsef, H. R., A. Ghobadi, M. Iranshahi, and M. Abdollahi. 2004. Antinociceptive effects of *Peganum harmala* L. alkaloid extract on mouse formalin test. J. Pharm. Pharm. Sci. 7:65–69.
Natt, M. P., and C. A. Herrick. 1952. New blood diluent for counting the erythrocytes and leucocytes of the chicken. Poult. Sci. 31:735–738.
Orskov, I., F. Orskov, E. Jann, and K. Jann. 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. 41:667–719.
Prakash, S. K. 2006. Effects of herbal extracts towards microbicidal activity against pathogenic *Escherichia coli* in poultry. Int. J. Poult. Sci. 5:259–261.
Prashanth, D., and S. John. 1999. Antibacterial activity of *Peganum harmala*. Fitoterapia 70:438–439.
Ríos, J. L., and M. C. Recio. 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100:80–84.
Saenz, Y., M. Zarazaga, L. Brinas, M. Lantero, F. Ruiz-Larrea, and C. Torres. 2001. Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. Int. J. Antimicrob. Agents 18:353–358.
Saify, Z. S., J. Farhad, N. Mushtaq, F. Noor, S. Akhtar, M. Arif, B. S. Naqvi, and M. H. Shoaib. 2005. Antibacterial activity of 1-methyl-7-methoxy-beta-carboline and its phenacyl and coumarine analogues. Pak. J. Pharm. Sci. 18:39–41.
Shahverdi, A. R., H. R. Monsef-Esfahani, B. Nickavar, L. Bitarafan, S. Khodaee, and N. Khoshakhlagh. 2005. Antimicrobial activity and main chemical composition of two smoke condensates from *Peganum harmala* seeds. Z. Naturforsch., C. J. Biosci. 60:707–710.
Sharaf, M., M. A. el-Ansari, S. A. Matlin, and N. A. Saleh. 1997. Four flavonoid glycosides from *Peganum harmala*. Phytochemistry 44:533–536.
Sobhani, A. M., S. A. Ebrahimi, and M. Mahmoudian. 2002. An in vitro evaluation of human DNA topoisomerase I inhibition by *Peganum harmala* L. seeds extract and its beta-carboline alkaloids. J. Pharm. Pharm. Sci. 5:19–23.
Stipkovits, L., K. Lapis, M. Hidvegi, E. Kosa, R. Glavits, and A. Resetar. 2004. Testing the efficacy of fermented wheat germ extract against *Mycoplasma gallisepticum* infection of chickens. Poult. Sci. 83:1844–1848.
Swarup, H., S. Arora, and S. C. Pathak. 1986. Laboratory Techniques in Modern Biology 2nd ed. Kalyani Publ., New Delhi, India.
Varley, H. 1975. Practical and Clinical Biochemistry, 4th ed. Arnold-Heinemann Publ. (India) Private Ltd., India.
Viveros, A., A. Brenes, I. Arija, and C. Centeno. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult. Sci. 81:1172–1183.
Zantop, D. W. 1997. Biochemistries. Pages 115–129 in Avian Medicine: Principles and Applications. B. W. Ritchie, G. J. Harrison, and L. R. Harrison, ed. Wingers Publ. Inc., Lake Worth, FL.