

In-Vitro and In-Vivo Antimicrobial Activity of Five Medicinal Plants against Virulent *Escherichia coli* O157:H7 Strain Harboring Shiga Toxin Gene

Asfand Yar Khan¹, Syed Saleem Ahmad^{1*}, Muhammad Avais¹ and Kamran Ashraf²

¹Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan

²Department of Parasitology, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan

ABSTRACT

This study aimed to determine the anti-bacterial efficacy of 5 different medicinal plant extracts against Shiga toxin (*E. coli* O157:H7) based on the *in-vitro* and *in-vivo* trial. A total of twenty-one (n=21) PCR-confirmed isolates were selected for detection of virulent genes *in-vitro* trial (zone of inhibition). For *in-vivo* trial, forty-two (n=42) day-old chicks were divided into seven equal groups randomly (n=6 birds/group), and were classified as: G1 (*Azadirachta indica* @ 18.25 mg/ml), G2 (*Melia azedarach* @ 15.75 mg/ml), G3 (*Withania coagulans* @ 25 mg/ml), G4 (*Nigella sativa* @ 30 mg/ml), and G5 (*Calotropis procera* @ 10 mg/ml) were taken as treatment groups, while G6 as a negative control, and G7 as a positive control. The results showed that 47.6% serovars were positive for the virulent gene (*Stx-1*). Moreover, the highest zone of inhibition was observed in G5 (17.1 ± 0.60 mm, P<0.05) as compared to other groups, which indicated high anti-bacterial efficacy on *in-vitro* basis. The survival rate and weight gain of chicks were significantly higher (P<0.05) in G5 compared with all other groups. However, none of the medicinal plant extracts affect liver function and blood parameters. Finally, in conclusion, *C. procera* was found to be highly effective to protect birds against *E. coli* O157:H7 infection.

Article Information

Received 09 October 2022

Revised 28 November 2022

Accepted 25 December 2022

Available online 09 June 2023
(early access)

Published 07 October 2024

Authors' Contribution

Conception: AY, SS. Methodological approach: AY, MA. Critical examination: AY, SS, MA, and KA. Writing initial draft preparation: AY, SS. Writing, assessment, and proofreading: AY, SS, and MA.

Key words

E. coli O157:H7, Shiga toxin-1, Antimicrobial activity, Medicinal plants, *Calotropis procera*

INTRODUCTION

Diarrhoea is one of the most frequent conditions in dogs (Ramos *et al.*, 2021), and is caused by various infectious agents such as viruses, protozoa (Gülersoy *et al.*, 2022) and bacteria (Candellone *et al.*, 2020). *Escherichia coli* is the major causative agent in causing diarrhoea in puppies (Algammal *et al.*, 2022), which is divided into intra-intestinal and extra-intestinal pathogenic serotypes (Sadeq *et al.*, 2018). The most common is enterohaemorrhagic serotype, subdivided into shigatoxigenic and verotoxigenic (Kiranmayi *et al.*, 2010). The most important serovar is *E. coli* O157:H7 (Abdulrazzaq *et al.*, 2021). It causes fatal

diseases in dogs, including hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Kwon and Cho, 2015; Boland *et al.*, 2013). Therefore, the accurate diagnosis of this serovar of *E. coli* is mandatory.

Medicinal plants are mainly used for human health. It had been used for the treatment of various diseases due to the presence of their phytochemical constituents (Fullerton *et al.*, 2011). According to WHO, 80% of communities in developing countries are using traditional medicines (Lee *et al.*, 2019). The wide use of medicinal plants as an alternative to conventional drugs is due to low anti-microbial resistance as compared to conventional drugs (Owolabi *et al.*, 2017). Plant-based therapeutics are biodegradable and harmless to the environment (Munir *et al.*, 2017). Experimentally it has been proven that products obtained from medicinal plants are the most economical and active natural antibiotics (Qureshi *et al.*, 2019).

Various plants have been used as antibacterial agents including *Azadirachta indica* (Parihar and Balekar, 2016), *Melia azedarach* (Khan *et al.*, 2022), *Nigella sativa*, *Withania coagulans* (Nwokafor *et al.*, 2020), and *Calotropis procera* (Kamruzzaman *et al.*, 2013). These herbal extracts interfere with the functions of the cell wall,

* Corresponding author: vetsalim@uvas.edu.pk
0030-9923/2024/0006-2919 \$ 9.00/00



Copyright 2024 by the authors. Licensee Zoological Society of Pakistan.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

cell membrane, and protein synthesis and lead to leakage in cytoplasmic bacteria, and finally inhibit the growth of bacteria (Dutta *et al.*, 2015). Despite the beneficial effects of these plant extracts, no study is reported against *E. coli* O157:H7 infection particularly in canines. Therefore, this study was designed to assess the antimicrobial activities of the cold aqueous extracts of *A. indica*, *M. azedarach*, *N. sativa*, *W. coagulans*, and *C. procera* by using various *in-vitro* and *in-vivo* methods.

MATERIALS AND METHODS

Ampilification of *E. coli* O157:H7 virulent genes

A total of 21 PCR-confirmed *E. coli* O157:H7 isolates were selected for the molecular detection of virulent genes (Shiga-toxins; *Stx-1* and *Stx-2*) through PCR by using the following primers:

Stx-1 (140 bp fragment)
F: ACCCTGTAACGAAGTTTGCG
R: ATCTCATGCGACTACTTGAC) and
Stx-2 (346 bp fragment)
F: TTAACCACACCCACGGCAGT
R: GCTCTGGATGCATCTCTGGT.

The DNA was extracted by using a commercial DNA extraction Kit (Gene JET Genomic DNA Purification Kit; Thermo Scientific, USA). PCR was conducted as per the protocols already described (Khan *et al.*, 2022).

Table I. Names of the plants and their parts used in the this study.

Name of plants (Common name)	Voucher No.
Leaves of <i>Azadirachta indica</i> (Neem)	GC. Herb. Bot. 3668
Leaves of <i>Melia azedarach</i> (Bakain)	GC. Herb. Bot. 3669
Leaves of <i>Calotropis procera</i> (Aak)	GC. Herb. Bot. 3672
Seeds of <i>Nigela sativa</i> (Kalonji)	GC. Herb. Bot. 3670
Seeds of <i>Withania coagulans</i> (Paneer bootie)	GC. Herb. Bot. 3671

Medicinal plants extracts

The experimental medicinal plants (*A. indica*, *M. azedarach*, *C. procera*, *N. sativa*, and *W. coagulans*) were procured from the local market of district Lahore, Pakistan, and were identified and authenticated by the Department of Botany in the Government College University, Lahore, Pakistan (Table I). The leaves of plants were dried for fifteen days and were grinded in an electric blender to form powder. Approximately 100 g/750 ml of solvent (double-distilled water) was extracted for 72 h on a vortex shaker (dathan scientific). A rotary evaporator was used to evaporate the filtrate at 37°C under reduced pressure. The pure yield was determined and stored at 4°C until further use.

Bacterial strain and culture medium

In this study, ten PCR-confirmed Shiga toxin-producing *E. coli* O157:H7 isolates were used. The strain was cultivated at 37°C in Luria broth (LB) and was preserved at -80°C in LB containing 15 % glycerol. The bacterial culture was prepared by inoculating bacteria into a tube containing LB and then kept overnight at 37°C before further use.

Well diffusion assay

Different dilutions of extracts such as 400 mg/ml, 300 mg/ml, 200 mg/ml, and 100 mg/ml (Torkan *et al.*, 2016) were prepared in distilled water. Ciprofloxacin 5µg was added as a positive control and 5 % DMSO was used as a negative control (20 ml). Mueller Hinton agar was poured in petri-plates and allowed to solidify. Plates were dried and 50 µl of bacterial suspension containing 9×10^9 cells/ml was poured into each plate and spread uniformly. The plates were allowed to dry for 5 min under sterile conditions. 30 µl of the extract was infused into one of the 8 mm diameter wells on each plate's agar surface. The plates were then incubated for 24 h at 37 °C, and the zone of inhibition was measured in mm.

Determination of minimum inhibitory concentration (MIC)

The broth microdilution method was used for MIC. In brief, 50 µl of Muller Hinton broth was filled from 1st well to 12 well of the microtitration plate. Then *E. coli* O157:H7 with the standard dilution of McFarland 9×10^9 cells/ml was filled from well 1 to 11. In the last, 100 mg/ml of cold aqueous extracts of the above plants were poured from well 1 to 10 of the microtitration plate and incubated at 37 °C for 24 h before and after the incubation the optical density (OD) values were taken. The wells 11 and 12 were used as the negative and positive control, respectively. The microtitration plate was then incubated for 24 h at 37°C. Turbidity in the negative control well was used to measure bacterial growth. The MIC values were estimated by comparing the wells with no bacterial turbidity to the negative control well.

Determination of minimum bactericidal concentration (MBC)

MBCs were determined by plating 100 µl of samples from each MIC assay tube with growth inhibition on freshly prepared sterile Muller Hinton agar plates and aerobically incubating them at 37 °C for 24 h. During the incubation period, the MBC values were recorded as the lowest concentration of extract that did not allow any visible bacterial colony growth on the agar plates. These tests were repeated thrice (Bilal *et al.*, 2020).

Experimental design

Growth of *E. coli* O157:H7 containing Stx-1 gene (A field strain that was previously isolated and identified by PCR) was grown overnight at 37 °C in LB broth medium. Broth culture was then diluted with normal saline to obtain a concentration of 9×10^9 cells/ml (ID_{50} dose). A total of 42 (un-vaccinated) day-old broiler chicks were purchased from a reputable hatchery and were used to determine the protective dosage of *Azadirachta indica*, *Melia azedarach*, *Withania coagulans*, *Nigella sativa*, and *Calotropis procera*. The birds were fed balanced commercial starter and growing rations (21% and 18%, respectively) and provided clean water, without using any antibacterial agent until the end of the experimental period. Birds were reared under open housing system under strict hygienic conditions. The chicks were divided into seven groups; Groups 1 to 5 were the treatment groups, groups 6 and 7 served as a negative and positive control, respectively. The infected groups (G1, G2, G3, G4, G5, G7) were kept in one room while the birds of G6 (Uninfected) were reared at a distance in another room with the similar housing and environmental conditions. Every chick (except the negative control group) was challenged with 1 ml of the inoculum orally after seven days of age. Group 7 (infected but untreated chicken) received no treatment during the experiment. G6 (negative control) received placebo only. G1 was treated with *A. indica* @ 18.25 mg/ml, G2 with *M. azedarach* @ 15.75 mg/ml, and G3 with *W. coagulans* @ 25 mg/ml, G4 was treated with *N. sativa* @ 30 mg/ml. while G5 was treated with *C. procera* @ 10 mg/ml. The groups (1-5) received treatments for five consecutive days (approx. 1ml orally of above said doses each day) after 24 h of the infection with *E. coli* O157:H7 containing Stx-1. All the chicks were kept under daily observation up to 10 days for clinical signs, mortalities, haemato-biochemical alterations, histopathological changes, and growth performance was also measured up to the 22nd day till the end of the experiment.

Haemato-biochemical parameters

Serum biochemical parameters alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) were assessed using commercial kits. While for hematological parameters like red blood cells (RBCs), Natt and Herrick method (Natt *et al.*, 1952) was used, and for hemoglobin estimation, Drabkins method (Balasubramaniam *et al.*, 1992) was practiced.

Histopathological examination

Histopathology was performed to observe the anti-microbial effects of medicinal plants against *E. coli* O157:H7 contained Shiga toxin in broiler birds

and compared with an infected group. At the end of the experiment, all the remaining birds were slaughtered from each group, and organs (liver, heart, spleen, and intestine) were collected in a jar having 10% buffered formalin and left for a week for hardening. Then processed for histopathological examination by using standard protocol.

Statistical analysis

Data were statistically analyzed through SPSS version 22. The data on the percentage of presence of virulent genes and survival percentages analyzed by using the chi-square (χ^2) test. However, the data of MIC, MBC, bird growth performance, liver function, and blood parameters were analyzed by using a two-way analysis of variance (ANOVA).

RESULTS

The results revealed that a total of 10 (47.6 %) isolates (Table II) were positive for the *Stx-1* gene and none of the isolates was positive for the *Stx-2* gene. However, there were 11 (52.3%) isolates were negative for both virulent genes.

Table II. Virulent genes found in *E. coli* O157:H7 isolated from canine diarrheic pups.

No. of genes	Genes	No. of <i>E. coli</i> O157:H7 harboring genes	%
One gene	<i>Stx-1</i> ^a	10	47.6 ^{***}
	<i>Stx-2</i> ^b	0	0
Two genes	<i>Stx-1</i> + <i>Stx-2</i>	0	0
None of the two genes	-	11	52.3

^aStx-1, Shiga toxin 1 gene, ^bStx-2, Shiga toxin 2 gene, (** P<0.0001; X²=16.51) significance between Stx-1 and Stx-2. ++ shows significance between one and two genes (++ P<0.0001; X²=14.82).

The results of antibacterial activity (zones of inhibition) are shown in Figure 1 The result showed that the zone of inhibition was significantly (P<0.05) higher in the control group (20.1±1.10 mm) compared to the treatment groups. The zone of inhibition was significantly increased as the dose of treatment groups increased, so that the zone of inhibition was highest at 400 mg/well in all the treatment groups. However, the *C. procera* showed higher efficacy against *E. coli* irrespective of treatment dose. The results indicated that the antimicrobial efficacy of the extracts was concentration-dependent.

The results of MIC and MBC assays are presented in Table III. The results showed that there was a significant difference in MIC and MBC values between *C. procera* (10

and 75.75, respectively; $P < 0.01$) and all other treatment extracts. These findings indicated that *C. procera* is a potent antibacterial agent than other herbs

Table III. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cold aqueous extracts of different plant extracts against *E. coli* O157:H7. Common letters show significant differences at $p < 0.05$.

Plants	MIC (mg/ml)	MBC (mg/100 μ l)
<i>A. indica</i>	18.25	125.50a
<i>M. azedarach</i>	15.75	92.75a
<i>W. coagulans</i>	25	>150a
<i>N. sativa</i>	30	275a
<i>C. procera</i>	10	75.75a

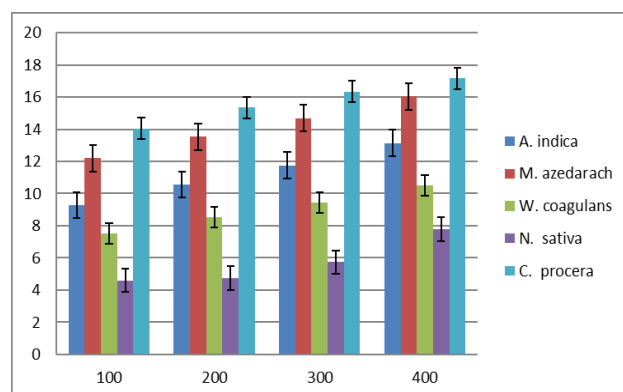


Fig. 1. Zones of inhibition induced by cold aqueous extracts of five medicinal plants against *E. coli* O157:H7 (All the values are expressed in mean $\pm$ standard error).

Feed intake, body weight, weight gain, and feed conversion ratio of broiler birds treated with medicinal compounds are shown in Table IV. The results showed that a significant difference ($P \leq 0.05$) was observed in feed intake among birds of experimental groups. The highest feed intake was observed in G 6 (887.21 g) during the experimental trial as compared to other treatment groups. However, the lowest feed intake was observed in G 7 group. The findings with respect to final body weight revealed that the birds from the G1, G2, and G5 gained significantly ($P < 0.05$) more weight than G3, G4, G6, and G7.

The survival rates of birds post-infection after treatment with herbal extracts are depicted in Figure 2. The results showed that there was a significantly higher survival rate in G6 and G5 (100%; $P < 0.01$) than control and treatment groups (G1-4). In G7 (control positive),

100 % of the birds died after 18 h of incubation (oral administration of *E. coli* O157:H7). The herbs had no impact on various liver function and blood parameters (ALP, AST and ALT, RBCs, HB, Table V).

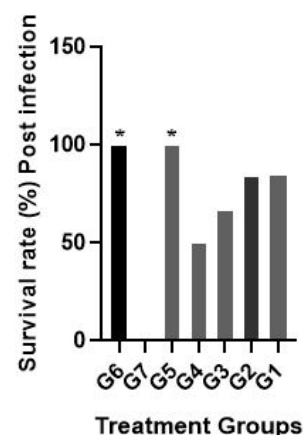


Fig. 2. The survival percentage of treated groups post infection. Marked * showed a significant higher survival rate in G6 and G5 (100 %, $P < 0.01$) than control and G1-4 treatment groups.

During the histopathological evaluation of G6 birds, all the organs were observed normal and didn't exhibit any change. In G7, the birds showed blunting, thickening and abnormal elongation of villi with heavy infiltration of inflammatory cells, and disruption in the apical surface of the intestine, eighteen h post-infection. Moreover, infiltration of lymphocytes, degeneration of myocytes, and extensive presence of inflammatory cells were observed in the heart, along with severe pathological lesions in the liver of the infected birds. In G1, 2, and G5, mucus attachment in the epithelium of the intestine, congestion of blood vessels, necrosis, infiltration of the inflammatory cells, and abnormal elongation of glands along with disruption in some parts of the caecum were observed. In G3, disruption of villi, elongation of glands, and lymphocytic proliferation of caecum along with severe necrosis, haemorrhages, and inflammation were noticed. While in G4, superficial sloughing of epithelial mucosa of the intestine, proliferation of monocytes, and lymphocytes were observed in the caecum. All the groups were observed with normal hearts. In the liver examination, mild degeneration of hepatocytes with an increased population of lymphocytes and macrophages was observed in G1. In G2 and 3, necrosis of hepatocytes and severe degeneration were noticed. In G4, severe degeneration in lymphocytes and pre-dominant lymphoblast were observed. While G5 tissues were found normal.

Table IV. Growth performance of broiler birds treated with medicinal compounds.

	Initial body weight (n=6)	Final body weight (n=6)	Weight gain (n=6)	Feed intake (n=6)	FCR (n=6)
Group 1	36.10 ± 1.70	548.89±0.90 ^{ab}	542.30±0.87 ^b	850.10±51.45 ^a	1.56±0.12 ^{ab}
Group 2	36.33 ± 0.94	574.30±0.87 ^b	518.80±0.81 ^b	851.10±63.51 ^a	1.64±0.11 ^{ac}
Group 3	35.47 ± 0.40	521.10±1.23 ^{ac}	484.20±1.12 ^c	855.65±52.30 ^a	1.76±0.05 ^a
Group 4	35.52 ± 0.42	480.50±2.80 ^c	453.60±1.097 ^c	846.20±138.50 ^b	1.86±0.02 ^a
Group 5	36.12 ± 0.52	615.00±0.78 ^a	575.62±0.77 ^a	860.31±56.27 ^a	1.49±0.01 ^b
Group 6	36.95 ± 0.93	541.11±2.65 ^c	434.20±1.01 ^c	840.21±47.24 ^b	1.93±0.06 ^a
Group 7	35.00 ± 0.10	219.21±1.35 ^a	190.15±0.07 ^a	90.01±22.01 ^a	0.47±0.02 ^b
p-value	0.5414	<0.0001	<0.0001	0.0121	0.0223

All the values are expressed in mean ± standard error. Common letters show non-significant differences at $p > 0.05$ whereas Different letters a-c within a column show significant differences when $p \leq 0.05$. FCR is feed conversion ratio. Group 1, *Azadirachta indica* treated group; Group 2, *Melia azadirach* treated group; Group 3, *Withania coagulans* treated group; Group 4, *Nigella sativa* treated group; and Group 5, *Calotropis procera* treated group; Group 6, negative control; Group 7, Positive control.

Table V. Blood and liver function profile of birds treated with medicinal plants.

	G1	G2	G3	G4	G5	G6	G7	p value
RBCs	4.59 ± .05	4.90 ± .50	3.71 ± 1.50	3.58 ± .80	4.90 ± .01	4.12 ± 5.1	2.76 ± 3.2	0.2
Hb	10.10 ± 1.21	12.10 ± 4.57	8.70 ± 1.21	8.30 ± 2.81	13.50 ± 2.50	9.89 ± 1.31	6.42 ± 1.05	0.08
ALP	15.50 ± .01	17.75 ± .61	14.15 ± .35	14.0 ± .75	18.22 ± 1.70	18.80± 3.67	11.75±2.10	0.3
AST	11.12 ± 1.10	13.21 ± 1.40	9.70 ± 3.30	10.65± 0.90	12.7 ± 1.22	13.12 ± 1.97	6.10 ± 1.10	0.1
ALT	58.0 ± 9.0	67.19 ± 12.0	51.50 ± 10.1	65.10± 12.0	59.48 ± 54.3	62.05 ± 32	45.20±9.05	0.6

For statistical details and details of groups, see Table IV. RBC, red blood cell; Hb, hemoglobin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

DISCUSSION

In the present study, the PCR revealed that the *Stx-1* gene is the most predominant virulent gene associated with *E. coli* O157:H7 isolated from diarrhoeic dogs. The pathogenicity of *E. coli* O157:H7 is directly attributed to its virulence factors (*Stx-1* and *Stx-2* genes) resulting destruction of eukaryote ribosome and inhibiting protein biosynthesis of the host (Algammal *et al.*, 2022). These findings agreed with Rahman *et al.* (2021) who reported that most of the *E. coli* O157:H7 isolates herboard *Stx-1* genes. In the present study, the prevalence of Shiga toxin-1 gene (47.6 %) is almost similar to that reported by the previous study (43.7 %) of Okwu *et al.* (2020). Moreover a lower prevalence of Shiga toxin-1 gene (33.3 %) was documented by Dhanki *et al.* (2018). The possible reason for variation in the prevalence of Shiga toxin-1 gene might be attributed to the different exposure of the dogs to the organism.

The present study confirmed the inhibitory effect of cold aqueous extract of leaf of *C. procera* through agar well diffusion method against *E. coli* O157:H7. In previous reports, the absence of inhibitory effect of sequentially extracted hot (water bath), methanolic extracts of *C. procera*

leaf against disc diffusion method was shown (Hossain *et al.*, 2021). The contradiction between previous and current studies results might be due to differences in extraction method, antibacterial activity method, or both. The past studies stated that some of the active biomolecules may escape from the extract as a result of the high-temperature treatment during the water bath extraction (Kayath *et al.*, 2022). As the free hydroxyl groups present in the disc may prevent the diffusion of cationic polar molecules, the agar well diffusion method might be superior to the disc diffusion method. The results of the current study proved that *M. azedarach* had the antibacterial inhibitory activity. A few earlier studies (Tavakkoli *et al.*, 2021), also documented that *M. Azedarach* contains antibacterial substances. Our study is inconsistent with the findings of Haq *et al.* (2020) and Singh and Kumar (2018) who stated that the leaves of *M. azedarach* are more effective against Gram-negative strains of bacteria than Gram-positive. The earlier studies suggested that *W. coagulans* exhibited moderate activity against *E. coli* (Bilal *et al.*, 2020) which support our study. The current study showed resistance for *E. coli* O157:H7 to cold aqueous extracts of *N. sativa*. Several investigators (Sharma and Kharel, 2019) had also reported that methanolic extracts of *N. Sativa* were

ineffective against *E. coli*, whereas few researchers (Yang and Rothman, 2004; Carvalho *et al.*, 2016; Subramaniam *et al.*, 2020; Fullerton *et al.*, 2011) found the antimicrobial activity of *N. sativa*. This discrepancy might be possibly due to difference in the characteristics of bacterial strains and difference in solvent extraction.

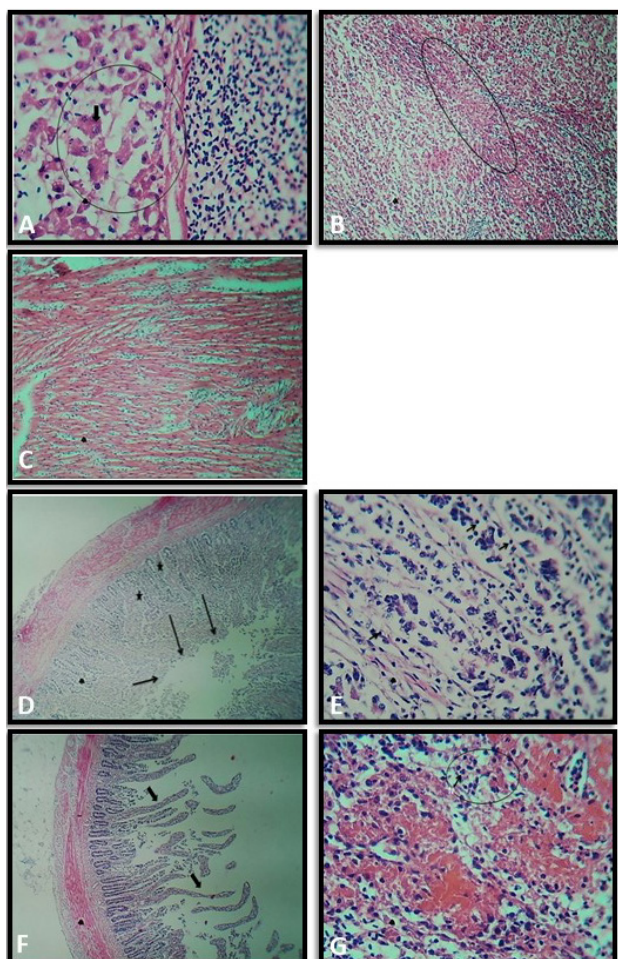


Fig. 4. Histo-pathological changes in liver (A, B), broiler heart (C), intestines (D, E, F) and spleen (G).

The encircled area in A (positive control) shows necrosis with empty spaces along with karyorrhexis and diffused congestion in the liver. The encircle area in B shows the diffused congestion in the liver. C Shows extensive presence of inflammatory cells in the myocarditis. D shows disrupted villi (arrows) while the stars in E indicated elongation of the glands of the cecum and lymphocytic proliferation (arrows) in cecum of *W. coagulans* group. Photomicrographs of broiler intestine and spleen group 4. F shows abnormal elongation of the villi of intestine of *Nigella sativa* treated group. Encircled area in G showed the infiltration of inflammatory cells, lymphocytes and neutrophils in spleen.

MIC refers to the lowest concentration of an antimicrobial agent that prevents the growth of the pathogen. High values of MIC are an indication of limited antibacterial efficacy. Current results are in agreement with the reports of various researchers, that the MIC of plants has been an important tool in determining the antimicrobial potential of plants (Abdulrazzaq *et al.*, 2021). The findings of the current study showed a significantly low (10 mg/ml) MIC value for *C. procera* against *E. coli* O157:H7. Similar findings were observed by Bilal *et al.* (2020) for *E. coli*. The plausible reason may be the similar dose rates or constituents in the extract of herbs. 28 mg/ml MIC of aqueous extract of *C. procera* against *E. coli* was documented in a previous study (Kishor *et al.*, 2020), which was slightly higher than our findings. The findings of the current study showed that the MIC activity of *M. azedarach* against *E. coli* O157:H7 was 15.75 mg/ml concentration. The results of the present study are incompatible with the past study of Kwon and Chao (2015) who reported 47.8 mg/ml MIC for aqueous extract of *M. azedarach* against *E. coli*. The findings of this study for MIC of *A. indica* extract against *E. coli* O157:H7 was 18.25 mg/ml, which are in agreement with Parihar and Balekar (2016). Our results for MIC for *W. coagulans* are supported by Sharma and Kharel (2019) and Zahid *et al.* (2020).

The feeding behaviour of our study showed that birds were significantly affected by herbs. *C. procera* treated group had the highest final weight and total weight gain. The increase in weight gain may be due to the highest amount of carbohydrate, ash, and protein found in *C. procera* (Rahman *et al.*, 2021; Okwu *et al.*, 2020). The results of our findings for *M. azedarach* disagreed with Tavakkoli *et al.* (2021) who reported that aqueous extract of *M. azedarach* significantly decreased body weight, final weight gain, and relative growth rate in birds. The incongruity in results may be due to difference in dose rate, extract consistency, and environmental condition. The performance of birds in our study by feeding *A. indica* showed significantly better performance as compared to the control healthy group. These results coincide with those of Owolabi *et al.* (2017), Qureshi *et al.* (2019) and Parihar and Balekar (2016). On the other hand, the present study opposed the findings of Munir *et al.* (2017).

Severe pathological lesions were reported in the current study in the liver of infected birds. Our findings in terms of infected liver birds are in accordance with those of Torkan *et al.* (2016) and Samuel and Sudi (2020) who concluded that the liver had the potential to absorb a large concentration of Shiga toxin travelled through the hepatic portal system.

In the present study, the results of histopathological evaluation agreed with those of Khan *et al.* (2022) who

demonstrated that inflammatory reaction in the intestine is due to the strain that survives in the gastrointestinal environment and colonize in the intestinal tissues. In heart, infiltration by the increasing number of lymphocytes, degeneration of myocytes, and extensive presence of inflammatory cells observed in the present study are corroborated by Zahid *et al.* (2020) who reported that *E. coli* O157:H7 produces pathological lesions in cardiac muscle fibers.

The results of the present study revealed that the birds treated with *C. procera* showed no significant difference ($p>0.05$) for ALT, ALP, AST, RBCs, and Hb. The present study disagrees with Derbal and Diar (2019) who stated that leaves of *C. procera* showed harmful effects on albino rats. Our research is consistent with the findings that *A. indica* leaf meal in the diet of broilers had no adverse effects on liver function parameters (Kiranmayi and Krishnaiah, 2010) and in contrast with Fullerton *et al.* (2011) who observed a significant difference ($P<0.05$) in blood profile of chickens. In the current study, the performance of birds by treating *A. indica* showed significantly better performance as compared to the control healthy group which coincides with the findings of Okwu *et al.* (2020) and Kayath *et al.* (2022) and opposes the findings of Owolabi *et al.* (2017), Viera *et al.* (2020) and Ali *et al.* (2021) who had observed no positive effects of the *A. indica* supplementation on the bird's performance. Our findings in terms of *W. coagulans* are in agreement with those of Kwon and Cho (2015) who concluded that *W. coagulans* serves as a potent alternative to synthetic compounds to improve broiler performance. Our findings in terms effect of *N. sativa* treatment on blood parameters is encouraged by Bilal *et al.* (2020). The liver function enzymes remained unaffected ($P>0.05$) by *N. sativa* as documented by Hossain *et al.* (2021) who stated that *N. sativa* had no adverse effect on (SGPT, SGOT, and ALP) and may be used as an alternative to antibiotics and as a growth promoter in broiler chickens.

CONCLUSION

The canine diarrhoeic puppies harbour Shiga toxins producing *E. coli* O157:H7. The higher prevalence of Stx-1 showed pathogenic potential of this strain. Aqueous extract of *C. procera* exhibited promising antibacterial activity against Shiga toxins producing *E. coli* O157:H7 among the medicinal plants used (*A. indica*, *M. azedarach*, *W. coagulans*, *N. sativa*) and may be a potential candidate in drug development for the treatment of Shiga toxins producing *E. coli* O157:H7 infection. In future, the detailed investigations may be carried out to explore the mode of action, side effects of these medicinal plants, and

their safety index, before development and application of plant based drugs to counteract Shiga toxins producing *E. coli* O157:H7 infection.

ACKNOWLEDGMENTS

The authors acknowledge Miss Sajida Mushtaq (Head of Diagnostics) and Dr. Saba Rafique (Head of Research and Development) at SB Poultry Diagnostic Lab, Rawalpindi for helping in the optimization of PCR. The authors would like to thank the owner of the Dogs for their assistance during sample collection. The authors are also grateful to the Department of Veterinary Medicine staff.

Funding

This study was financially supported by the Higher Education Commission (HEC) of Pakistan for funding us through the National Research Programme for Universities (NRPU) project # (9719/NRPU/R & D/HEC)

IRB approval

The research trial of the study was approved by the Institutional Review Board of UVAS, Lahore, Pakistan. (Letter No. 7522/date: 18-07-2019).

Ethical statement

The experimental design and protocols of this study were ethically approved by the Institutional Ethical Committee, UVAS Lahore, Pakistan (Letter No. 949/date: 19-09-2019).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

- Abdulrazzaq, K.M., Owain, M.S., Majeed, H.M. and Al-Hyani, O.H., 2021. Molecular detection of rfbO157, shiga toxins and hemolysin genes for *Escherichia coli* O157:H7 from canine feces in Tikrit and Mosul cities. *Iraqi J. Vet. Sci.*, **5**: 325-329. <https://doi.org/10.33899/ijvs.2020.126831.1392>
- Algammal, M., Mabrok, M., Ezzat, M., Alfifi, J., Esawy, M., Elmasry, N. and El-Tarabili, M., 2022. Prevalence, antimicrobial resistance (AMR) pattern, virulence determinant and AMR genes of emerging multi-drug resistant *Edwardsiella tarda* in Nile tilapia and African catfish. *Aquaculture*, **548**: 737-643. <https://doi.org/10.1016/j.aquaculture.2021.737643>
- Ali, E., Islam, M.S., Hossen, M.I., Khatun, M.M. and

- Islam, M.A., 2021. Extract of neem (*Azadirachta indica*) leaf exhibits bactericidal effect against multidrug resistant pathogenic bacteria of poultry. *Vet. Med. Sci.*, **7**: 1921-1927. <https://doi.org/10.1002/vms3.511>
- Balasubramaniam, P. and Malathi, A., 1992. Comparative study of hemoglobin estimated by Drabkin's and Sahli's methods. *J. Postgrad. Med.*, **38**: 8. <https://doi.org/10.4103/1858-5000.157502>
- Bien, J., Sokolova, O. and Bozko, P., 2012. Role of uropathogenic *Escherichia coli* virulence factors in development of urinary tract infection and kidney damage. *Prev. Vet. Med.*, **2012**: 681473. <https://doi.org/10.1155/2012/681473>
- Bilal, H., Ali, I., Uddin, S., Khan, I., Said, A., Rahman, M.U., Khan, A.M., Shah, A.B. and Ali, A., 2020. Biological evaluation of antimicrobial activity of *Calotropis procera* against a range of bacteria. *J. Pharmacogn. Phytochem.*, **9**: 31-35.
- Boland, K.G., Hayles, A.N., Miller, C.B., Kerr, T., Brown, W.C. and Lahmers, K.K., 2013. Regional immune response to immunization with *Escherichia coli* O157: H7-derived intimin in cattle. *Clin. Vaccine Immunol.*, **20**: 562-571. <https://doi.org/10.1128/CVI.00743-12>
- Candellone, A., Cerquetella, M., Girolami, F., Badino, P. and Odore, R., 2020. Acute diarrhea in dogs: Current management and potential role of dietary polyphenols supplementation. *Antioxidants*, **9**: 7-25. <https://doi.org/10.3390/antiox9080725>
- Carvalho, A.C., Barbosa, A.V., Arais, L.R., Ribeiro, P.F., Carneiro, V.C. and Cerqueira A.M.F., 2016. Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. *Braz. J. Microbial.*, **47**: 150-158. <https://doi.org/10.1016/j.bjm.2015.11.005>
- Conrad, C.C., Stanford, K., McAllister, T.A., Thomas, J. and Reuter, T., 2014. Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces. *J. Microbial. Methods*, **105**: 22-30. <https://doi.org/10.1016/j.mimet.2014.06.020>
- Derbal, S. and Niar, A., 2019. Antibacterial activity of honey and *Nigella sativa* L. seed extracts against animal wound bacteria. *Int. J. Vet. Sci.*, **5**: 30-34.
- Dhanki, Z.T.M., Al-Enzy, A.F.M. and Al-Hamdani, A.A.Y., 2018. Effect of aqueous extract of *Melia azedarach* L, *Anastatica hierochuntic* and enrofloxacin antibiotic on live broiler performance. *Eurasia Proc. Sci. Technol. Eng. Math.*, **3**: 110-115.
- Dutta, P., Borah, M., Gangil, R. and Singathia, R., 2015. Gross/Histopathological impact of *Salmonella gallinarum* isolated from layer chickens in Jaipur and their antibiogram assay. *Int. J. Adv. Vet. Sci. Tech.*, **4**: 153-159. <https://doi.org/10.23953/cloud.ijavst.179>
- Fullerton, M., Khatiwada, J., Johnson, J.U., Davis, S. and Williams, L.L., 2011. Determination of antimicrobial activity of sorrel (*Hibiscus sabdariffa*) on *Escherichia coli* O157: H7 isolated from food, veterinary, and clinical samples. *J. Med. Ed.*, **14**: 950-956. <https://doi.org/10.1089/jmf.2010.0200>
- Gülersoy, E., Maden, M., Parlak, T.M. and Sayin, Z., 2022. Comparative evaluation of selected serum and urine biomarkers in cats with interstitial and bacterial cystitis. *Vet. Clin. Pathol.*, pages Pages 1-9. <https://doi.org/10.1111/vcp.13174>
- Hammermuller, J., Kruth, S., Prescott, J. and Gyles, C., 1995. Detection of toxin genes in *Escherichia coli* isolated from normal dogs and dogs with diarrhea. *Can. J. Vet. Res.*, **59**: 265-270.
- Haq, I., Hafeez, A. and Khan, R.U., 2020. Protective effect of *Nigella sativa* and *Saccharomyces cerevisiae* on zootechnical characteristics, fecal *Escherichia coli* and hematopoietic potential in broiler infected with experimental colibacillosis. *Livest. Sci.*, **239**: 104119. <https://doi.org/10.1016/j.livsci.2020.104119>
- Hasan, M.S., Yousif, A.A. and Alwan, M.J., 2016. Detection of virulent genes in *E. coli* O157: H7 isolated from puppies and adult dogs by polymerase chain reaction. *Res. J. Vet. Pract.*, **4**: 1-6. <https://doi.org/10.14737/journal.rjvp/2016/4.1.1.6>
- Hossain, M.E., Siddiqua, M.T. and Hossain. M.M., 2021. Dietary inclusion of neem leaf powder on growth performance, blood biochemical parameters and profitability of broilers. *Bangladesh J. Vet. Anim. Sci.*, **50**: 114-122. <https://doi.org/10.3329/bjas.v50i2.58140>
- Hossain, M.S., Faruk, M.A.Z., Das, D. and Das, S., 2021. Effects of Neem (*Azadirachta indica*) and Tulsi (*Ocimum sanctum*) extract in the growth performance of broiler with economics of production. *J. Vet. Med. Anim. Sci.*, **4**: 10-66.
- Kamruzzaman, M., Bari, S.N. and Faruque, S.M., 2013. *In vitro* and *in vivo* bactericidal activity of Vitex negundo leaf extract against diverse multidrug resistant enteric bacterial pathogens. *Asian Pac. J. Trop. Med.*, **6**: 352-359. [https://doi.org/10.1016/S1995-7645\(13\)60038-3](https://doi.org/10.1016/S1995-7645(13)60038-3)
- Kayath, P., Datt, M., Kumar, L., Chopra, G. and Tatarwal, J.M., 2022. Effect of neem (*Azadirachta indica*) supplementation on hematological parameter of

- Kuroiler chicks. *Pharma. Innov.*, **11**: 1825-1829.
- Khafaji, M.N., Shlash, E.Y., Nayef, A.I. and Shaker, N.S., 2019. Protective effect of aqueous-methanol extract of *Melia azedarach* against paracetamol-induced hepatitis in rabbits. *Diyala J. Pl. Sci.*, **15**: 49-63. <https://doi.org/10.24237/djps.15.04.500D>
- Khan, A.Y., Ahmad, S.S., Avais, M. and Ashraf, K., 2022. Molecular prevalence with associated risk factors and haemato-serum electrolyte analysis of *E. coli* O157: H7 in Canine pups with diarrhoea. *Pak. Vet. J.*, **42**: 2074-7764.
- King, T., Lucchini, S., Hinton, J.C. and Gobius, K., 2010. Transcriptomic analysis of *Escherichia coli* O157: H7 and K-12 cultures exposed to inorganic and organic acids in stationary phase reveals acidulant- and strain-specific acid tolerance responses. *Appl. environ. Microbial.*, **76**: 6514-6528. <https://doi.org/10.1128/AEM.02392-09>
- Kiranmayi, C. and Krishnaiah, N., 2010. Detection of *Escherichia coli* O157:H7 prevalence in foods of animal origin by cultural methods and PCR technique. *Vet. World*, **3**: 13. <https://doi.org/10.5455/vetworld.2010.13-16>
- Kishor, K., Sahni, Y.P., Bhardwaj, J.K., Gautam, V. and Sawarkar, A., 2020. Effect of different fortifications of Panchgavya with *Nigella sativa* and *Asparagus racemosus* on growth performance parameters in poultry. *J. Pharmacogn. Phytochem.*, **9**: 1821-1825. <https://doi.org/10.20546/ijcmas.2020.910.254>
- Kwon, T. and Cho, S.H., 2015. Draft genome sequence of enterohemorrhagic *Escherichia coli* O157 NCCP15739, isolated in the Republic of Korea. *Genome. Announc.*, **3**: 15. <https://doi.org/10.1128/genomeA.00522-15>
- Lee, C., Kim, S.Y., Eum, S., Paik, J.H., Bach, T.T., Darshetkar, A.M., Choudhary, R.K., Quang, B.H., Thanh, N.T. and Choi, S., 2019. Ethnobotanical study on medicinal plants used by local Van Kieu ethnic people of Bac Huong Hoa nature reserve, Vietnam. *J. Ethnopharm.*, **231**: 283-294. <https://doi.org/10.1016/j.jep.2018.11.006>
- Majhool, A.B. and Zenad, K.H., 2021. Histopathological changes associated with infection of mice with shiga toxin producing *Escherichia coli* (STEC) O157: H7. *Annls Rom. Soc. Cell. Biol.*, **25**: 17486-17492.
- Melton-Celsa, A.R., 2014. Shiga toxin (Stx) classification, structure, and function. *Microbiol. Spectr.*, **2**: 2-42. <https://doi.org/10.1128/microbiolspec.EHEC-0024-2013>
- Miko, A., Rivas, M., Bentancor, A., Delannoy, S., Fach, P. and Beutin, L., 2014. Emerging types of Shiga toxin-producing *E. coli* (STEC) O178 present in cattle, deer, and humans from Argentina and Germany. *Front. Cell. Infect. Microbial.*, **4**: 78. <https://doi.org/10.3389/fcimb.2014.00078>
- Munir, T., Mohyuddin, A., Khan, Z. and Haq, R., 2017. Exploration of antibacterial potential of *Melia azedarach* L. *Sci. Inquiry Rev.*, **1**: 19-26. <https://doi.org/10.29145/sir/11/010103>
- Natt, M.P. and Herrick, C.A., 1952. A new blood diluent for counting erythrocytes and leucocytes of the chicken. *Poul. Sci.*, **31**: 735-738. <https://doi.org/10.3382/ps.0310735>
- Nwokafor, C.V., Udensi, C.G., Ogbonna, H.N., Udekwa, C.E., Nwankpa, U.D., Amanze, E.K., Chibuzor, W.N. and Okeke, K.C., 2020. Antimicrobial activities of moringa, neem and ginger plant extracts against bacteria associated with the spoilage of fruit juice. *South Asian J. Res. Microbial.*, **7**: 21-30. <https://doi.org/10.9734/sajrm/2020/v7i430179>
- Okwu, M.U., Imade, O.S., Akpoka, O.A., Olley, M., Izevbuwa, O.E. and Usman, N.A., 2020. Comparative evaluation of the antibacterial effects of *Moringa oleifera* and *Nigella sativa* on some clinical bacterial isolates obtained from Igbinedion University, Okada, Nigeria. *Biyoloji Bilimleri Araştırma Dergisi*, **13**: 20-27.
- Owolabi, A.O., Abah, K.A. and Oranusi, S.U., 2017. *In vitro* antimicrobial and antioxidant activity of *Carica papaya* and *Azadirachta indica* leaf and stem bark extracts on selected clinical isolates. *Ind. J. Sci. Technol.*, **6**: 209-220.
- Parihar, G. and Balekar, N., 2016. *Calotropis procera*: A phytochemical and pharmacological review. *Thai J. Pharm. Sci.*, **40**: 115-131.
- Pitout, J., 2012. Extra-intestinal pathogenic *Escherichia coli*: A combination of virulence with antibiotic resistance. *Front. Microbiol.*, **3**: 9. <https://doi.org/10.3389/fmicb.2012.00009>
- Puño-Sarmiento, J., Medeiros, L., Chiconi, C., Martins, F., Pelayo, J., Rocha, S. and Nakazato, G., 2013. Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. *Vet. Microbiol.*, **166**: 676-680. <https://doi.org/10.1016/j.vetmic.2013.07.007>
- Qureshi, S.A., Jahan, M., Lateef, T., Ahmed, D., Rais, S. and Azmi, M.B., 2019. Presence of gallic acid and rutin improve the hepatoprotective strength of Withaniacoagulans. *Pak. J. Pharm. Sci.*, **32**: 301-308.
- Rahman, M., Rahaman, M., Islam, M., Hossain, M., Mannan, M., Ahmed, M., Saldías, M., Akkol, E.K. and Sobarzo-Sánchez, E., 2021. Multifunctional

- therapeutic potential of phytocomplexes and natural extracts for antimicrobial properties. *Antibiotics*, **10**: 1076. <https://doi.org/10.3390/antibiotics10091076>
- Ramos, C.P., Diniz, A.N., Ribeiro, M.G., Paula, C.L., Costa, É.A., Sonne, L., Pereira, S.T., Lopes, C.E.B., Rennó, M.C., and Silva, R.O.S., 2021. Enteric organisms detected in feces of dogs with bloody diarrhea: 45 cases. *Top. Compan. Anim. Med.*, pp. 100549-100549. <https://doi.org/10.1016/j.tcam.2021.100549>
- Sadek, M., Saad, A.M., Nordmann, P. and Poiriel, L., 2022. Genomic characterization of an extensively drug-resistant extra-intestinal pathogenic (ExPEC) *Escherichia coli* clinical isolate co-producing two carbapenemases and a 16S rRNA Methylase. *Antibiotics*, **11**: 1479. <https://doi.org/10.3390/antibiotics11111479>
- Sadeq, J.N., Al-Mohamed, S.A. and Salim, R.M., 2018. Molecular identification of *E. coli* virulence factors genes, bfp and elt, in feces of diarrheic chickens. *Al-Qadisiyah J. Pure. Sci.*, **23**: 17-29. <https://doi.org/10.29350/jops.2018.23.2.734>
- Samuel, K., and Sudi, Y.I., 2020. Effects of *Calotropis procera* latex on biochemical and hematological parameters in albino rats. *Niger. J. Biotechnol.*, **37**: 94-100. <https://doi.org/10.4314/njb.v37i1.10>
- Sharma, K.R. and Kharel, R., 2019. Antibacterial, antidiabetic and brine shrimp lethality activities of some selected medicinal plants from Kavrepalanchok District of Nepal. *J. Inst. Sci. Technol.*, **24**: 57-62. <https://doi.org/10.3126/jist.v24i1.24629>
- Singh, P.K. and Kumar, A., 2018. Effect of dietary black cumin (*Nigella sativa*) on the growth performance, nutrient utilization, blood biochemical profile and carcass traits in broiler chickens. *Anim. Nutr. Feed. Technol.*, **18**: 409-419. <https://doi.org/10.5958/0974-181X.2018.00038.0>
- Subramaniam, G., Sivasamugham, L.A. and Yew, X.Y., 2020. Antibacterial activity of *Cymbopogon citratus* against clinically important bacteria. *S. Afr. J. Chem. Eng.*, **34**: 26-30. <https://doi.org/10.1016/j.sajce.2020.05.010>
- Tavakkoli, A., Mirakzehi, M.T., Saleh, H. and Yousefi, M., 2021. The effects of supplementation of *Withania coagulans* and α -tocopherol acetate in diets containing oxidised oil on growth performance, immune response and antioxidant indices in broiler chickens. *Arch. Anim. Nutr.*, **75**: 278-293. <https://doi.org/10.1080/1745039X.2021.1942765>
- Torkan, S., Bahadoranian, M.A., Khamesipour, F. and Anyanwu, M.U., 2016. Detection of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from diarrhoeic dogs in Iran. *Arch. Med. Vet.*, **48**: 181-190. <https://doi.org/10.4067/S0301-732X2016000200008>
- Vieira, Y.C., Silva, D.A., Marques, A.D.S., Santana, A.P., Murata, L.S. and Perecmanis, S., 2020. Detection of enterotoxin and adhesin genes of *Escherichia coli* strains isolated from feces of healthy dogs. *Acta Vet. Brasil.*, **14**: 1. <https://doi.org/10.21708/avb.2020.14.1.8921>
- Yang, S. and Rothman, R.E., 2004. PCR-based diagnostics for infectious diseases: Uses, limitations, and future applications in acute-care settings. *Lancet Infect. Dis.*, **4**: 337-348. [https://doi.org/10.1016/S1473-3099\(04\)01044-8](https://doi.org/10.1016/S1473-3099(04)01044-8)
- Zahid, R., Khan, J., Khuram, M. and Khan, M.A., 2020. Local medicinal plants of Pakistan in the fight against gastro-intestinal tract pathogenic bacteria. *Pak. J. Biotech.*, **17**: 173-182.