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Antimicrobial Action of Olive Leaf (*Olea europaea*) Extract and its Interaction with Antibiotics in *Salmonella gallinarum* Infected Broiler Chicks

Shah Nawaz[1,2], Farzana Rizvi[1]*, Muhammad Asif[3], Muhammad Shahzad Shafiq[1], Ayesha Ramzan[1], M.Z. Shakir[1,4], Sanobar Bibi[5], Samina Shabbir[6], Ali Asif[2], Md. F. Kulyar[2]* and Zeeshan Ahmad Bhutta[7]*

[1]*Department of Pathology, University of Agriculture Faisalabad, Pakistan*
[2]*Department of Clinical Veterinary Medicine, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, China*
[3]*Department of Veterinary Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[4]*Institute of Drug Discovery Technology, Ningbo University, China*
[5]*The Government Sadiq College for Women University, Bahawalpur, Pakistan*
[6]*Department of Chemistry, The Women University, Multan, Pakistan*
[7]*Laboratory of Veterinary Immunology and Biochemistry, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Republic of Korea*

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Authors' Contribution
SN and ZAB designed the experiment. SN, SS, MZS and AR performed animal and laboratory experiments. SN, MA and SB wrote original manuscript. MFK, SS and AA revised the manuscript. FR supervised the project. All authors read and approved the final manuscript.

Key words
***S. gallinarum*, Olive leaf extract, Antimicrobial resistance, Chicken**

ABSTRACT

Ethanolic extract of olive (*Olea europaea*) leaves contains phenolic compounds; oleuropein and ligstroside. The current study was designed to investigate the antibacterial property of *Olea europaea* leaf extract (OLE) (orally @10ml/l) alone and in combination with antibiotics in *Salmonella gallinarum* infected broiler chicks. A total of 125 one day old chicks were divided into 5 equal groups (25 chicks in each). Two groups were kept as negative and positive control, while others were supplemented with OLE, antibiotic, and OLE + antibiotic, respectively. On 14th day, experimental birds were infected with a field isolate of *S. gallinarum* (8.5×10^8 CFU/ml). The study revealed that morbidity and mortality rates were significantly lower in OLE supplemented groups. While there was a significant increase in body weight, erythrocytic count, hemoglobin concentration, and packed cell volume in OLE supplemented groups. Moreover, OLE was found with *in-vitro* zone of inhibition (18mm) against this bacterium. There was also a positive impact of OLE supplementation on total protein, albumin, and globulin concentrations than that of the rest of the groups. In conclusion, OLE has antimicrobial properties against *S. gallinarum* along with anabolic effect on the weight gain of broiler chickens and reduces the morbidity and mortality rate.

INTRODUCTION

Millions of people are suffering from poverty and inadequate nutrition resulting in energy deficient and immune compromised young ones in developing countries. Poultry eggs and meat are the vibrant source of animal protein (Farrell, 2013). The per capita protein requirement is 103g, while availability in Pakistan is 67g (Hussain *et al*., 2015). So, it is essential to improve the poultry production, but this sector facing numerous challenges. Infectious diseases are the most important impediment to its growth. Disease outbreak caused about 80% mortality of the parent flocks in northern areas of Pakistan (Hussain *et al*., 2015). However vast potential exists for the development of the poultry industry in Pakistan.

Salmonellosis is one of the major poultry diseases, specifically in developing countries with high ambient temperatures (Oliveira *et al*., 2000). *Salmonella* is a Gram negative intracellular bacteria grouped into motile

* Corresponding author: frizivi@yahoo.com, fakharealam786@hotmail.com, zee@cbnu.ac.kr
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and non-motile serovar. Non-host-specific (paratyphoid) *Salmonella* causes food born infections in humans and includes various motile serotypes, whilst host specific non-motile serotype includes the *S. gallinarum* and *S. pullorum* (Forshell and Wierup, 2006). Salmonellosis is a zoonotic disease with about 2500 serotypes and infects poultry, sheep, goat, pig, and cattle.

Salmonella enterica subspecies *Enterica serovar* (*Salmonella gallinarum*) causes fowl typhoid and is a disease of all ages of poultry birds. It is of most economic importance because of the high mortality, up to 90% (Sannat *et al*., 2017). Its eradication is highly challenging because of the transovarial contamination of eggs, and affected birds remain carriers throughout their life (Lim *et al*., 2011). It is vigorously treated by the irrational use of antibiotics, resulting in the development of multi-drug resistant genes of *Salmonella*. Therefore, it is essential to discover the alternate disease treatment methods (Sannat *et al*., 2017).

About 6000 years ago, olives were cultivated around the Mediterranean civilization, and the present production is over 23 million acres. Traditionally, its oil was used for lamp oil and is now used for food and medicinal purposes due to its tremendous results against many diseases. There are about 16 million trees/year, which belong to 19 classes of olive oil worldwide (Vossen, 2007). The olive tree (*Olea europaea*) is cultivated in temperate regions, covering more than 95% of the crop throughout the world (Oliveira *et al*., 2000). The olive tree has industrial and religious importance and is narrated as a tree full of the blessings in the Holy Quran and Holy Bible (Hashmi *et al*., 2015) .

The active ingredients of olive leaf include oleuropein, hydroxytyrosol, and flavonoids. The medicinal benefits of olive are because of these active ingredients (Charoenprasert and Mitchell, 2012). The biophenols of olive leaf extract act in two ways one of them is pharmacological action, and the other is the chemical mechanism of action. The chemical mechanism of action includes metal chelation, scavenging of reactive oxygen species, and as a reducing agent. While pharmacological action includes the enzyme modulation, interfering in cell division, signal transduction alteration, and gene expression modulation (Obeid *et al*., 2012). Through these two pathways, olive leaf extract plays an important role in the field of medicine.

It also reported that olive leaf reduce uric acid, cholesterol, and blood sugar in diabetic patients and also have antioxidant, antiviral, anthelmintic, anticancer, and antibacterial effects (El and Karakaya, 2009). Hence the project has been contrived to analyze the use of olive leaf extract as an alternative to antibiotics and its interaction with them to reduce morbidity and mortality in *Salmonella gallinarum* infected broiler birds.

MATERIALS AND METHODS

Experimental chicks

A total of 125 one day old Ross broiler chicks were used in this study. Chicks were purchased from the local breeder and raised under 23 h light, 75-80% relative humidity, and 32-24 °C temperature in decreasing manner from 1st to 5th week. Feed and water were provided on an *ad libitum* basis. Vaccine of Newcastle disease, infectious bronchitis, avian influenza, and infectious bursal disease was performed as per the established protocol of the University of Agriculture, Faisalabad, Pakistan.

Olive leave extract (OLE)

Olive leaves were collected from the botanical garden of the University of Agriculture Faisalabad. Leaves were dried under shade, and 10% ethanol OLE was prepared through a standardized extraction protocol described earlier (Gberikon *et al*., 2015).

Experimental design

Experimental birds were divided randomly into five equal groups (A, B, C, D, and E), 25 in each. Groups A and B were negative and positive control, respectively, while groups C and D were supplemented with ethanolic extract of olive leaf @10 ml/L of drinking water from 5-35th days of life (De Oliveira and Berchieri, 2005). Chicks of groups B, C, D, and E were challenged with 0.1ml of pure culture broth of *S. gallinarum* orally @ 8.2×10^8 CFU/ml at the 14th day of age. Birds of group D and E were treated with antibiotic (Florfenicol @30 mg/kg body weight) for five days after the appearance of clinical signs of fowl typhoid. After infection, birds were kept under observation for signs and symptoms. Blood was collected from wing vein with anticoagulant from five birds/group for hematological studies. After weighing slaughtering of five birds from each group was done on weekly bases up to 5th week of age. The visceral organs of slaughtered birds showing lesions were collected and stored in the neutral buffer for histological examination (Bancroft and Gamble, 2007). *S. gallinarum* was isolated from experimentally infected birds for antibiogram assay (Paul *et al*., 2017). The mortality percentage of each group was calculated at the end of the experiment.

Serological examination

Serum was collected from blood samples for serum biochemistry on each slaughtering. Total protein, albumin, and globulin concentration were determined by using a commercial kit. The concentrations of aspartate

aminotransferase (AST) and alanine aminotransferase (ALT), was also measured to check liver function through the commercial kit, ASAT (GOT) FS (IFCC mod.) Reference No. 126019910021 (Diagnostic System GmbH).

Antibacterial activity of OLE

The disc diffusion method was used to detect the possible antimicrobial activity of OLE against bacterial isolates of *S. gallinarum*. Blank paper discs of 6mm diameter (Whatman # 3 paper) were soaked with 20µl OLE (2mg/disc) and then placed on the Mueller Hinton Agar and XLD at which test organism was inoculated 1 h before @10^8 CFU/ml. Then plates were incubated at 37±.2C° for 48h. Some antibiotics were also used to compare their inhibitory action against *S. gallinarum* including gentamycin, florfenicol, ampicillin, ciprofloxacin, enrofloxacin, amikacin, and oxytetracycline.

RESULTS AND DISCUSSION

During study, clinical signs in infected birds were dehydration, ruffled feathers, diarrhea, anorexia, and respiratory distress. Similar clinical signs were observed in broiler breeder flock during an outbreak (Shivaprasad, 2000). Such clinical signs were also found in experimentally infected layer birds by *S. gallinarum* and in natural outbreaks (Biazus *et al*., 2017; Haider *et al*., 2013; Onuigbo *et al*., 2018). During an outbreak in the commercial layer, yellowish green diarrhea, pale mucous membrane and sudden death were reported (Kwon *et al*., 2010), while these signs and sudden death was not observed in current trail. The pattern of clinical signs and symptoms was found as highly sever in group 'B' chickens with almost all the signs mentioned above than group 'C' and group 'D' chickens with decreasing trend in the severity, respectively.

The prominent gross pathological lesions in *Salmonella* positive groups were as white necrotic foci on the liver, hepatomegaly, splenomegaly, and swollen kidney bronze discoloration of the liver with hepatomegaly, and splenomegaly with necrotic foci. Similar lesions were also noticed earlier (Shivaprasad, 2000). Thickening of hepatic capsule and hepatomegaly due to inflammatory cellular infiltration (ICI) and bile duct epithelial hyperplasia could be due to the direct mitogenic effect of bacterial endotoxin (Gheith, 2008). Bacterial clumps in hepatic parenchyma and accumulation of bile due to bile duct obstruction by bacterial accumulation could be the reason for bronze discoloration of the liver (Soufy *et al*., 2016). During a field study, white multiple foci were also observed on the liver and spleen in broiler breeder and commercial layers Kwon *et al*. (2010). Red spots on the surface of the lungs with thickening of the pericardium and increased turbid pericardial fluid were observed in experimentally infected broiler chicks with *S. gallinarum* (Soufy *et al*., 2016), but in the current study, thickening of the pericardium and turbid pericardial fluid was not found. Swollen liver and spleen with off-white to yellowish necrotic foci were witnessed in Japanese quail (Casagrande *et al*., 2014) .

As the pathogenesis of *Salmonella* concerns following engulfing, it penetrates through intestinal M-cells, then migrates through dendritic cells and subsequently enters circulation via mesenteric lymph nodes (Fig. 1) and results in appearance of above-mentioned pathogenic lesions.

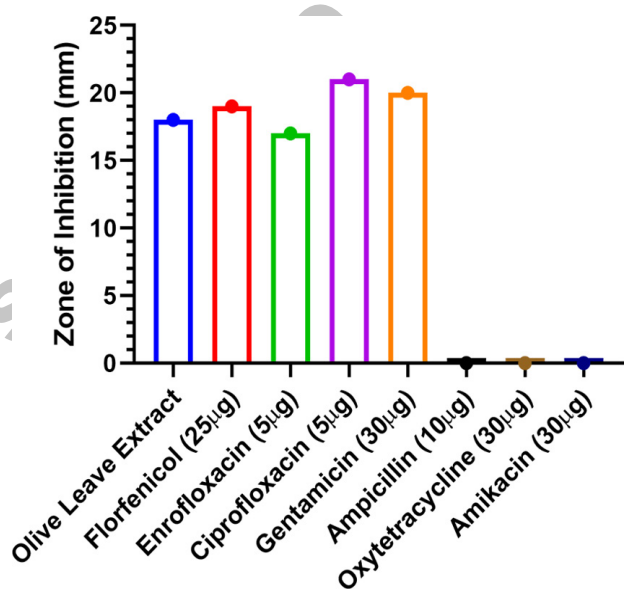

Fig. 1. Sensitivity of *S. gallinarum* against olive leave extract and antibiotics as indicated by zones of inhibition.

The disc diffusion method was used to check the *in-vitro* sensitivity of bacterial isolates of *S. gallinarum*. Blank paper discs were soaked in OLE and then placed on the Mueller Hinton Agar at which *S. gallinarum* was sprinkled one hour before. Some antibiotics were also used to check their inhibitory action against *S. gallinarum*: gentamycin, florfenicol, ciprofloxacin and enrofloxacin. The zone of inhibition shown by gentamycin, florfenicol, olive leave extract, enrofloxacin and ciprofloxacin are visible (Fig. 2). This *in-vitro* study confers the antibiotic activity of OLE. Moreover, the zone of inhibition of oxytetracycline, ampicillin and amikacin which was 0 mm indicates the development of antibiotic resistance in *S. gallinarum* against these antibiotics. In the current study, ethanolic extract of olive leaf exhibited antimicrobial action with

18mm zone of inhibition against *S. gallinarum*. The results are in line with the early study (Keskin *et al*., 2012).

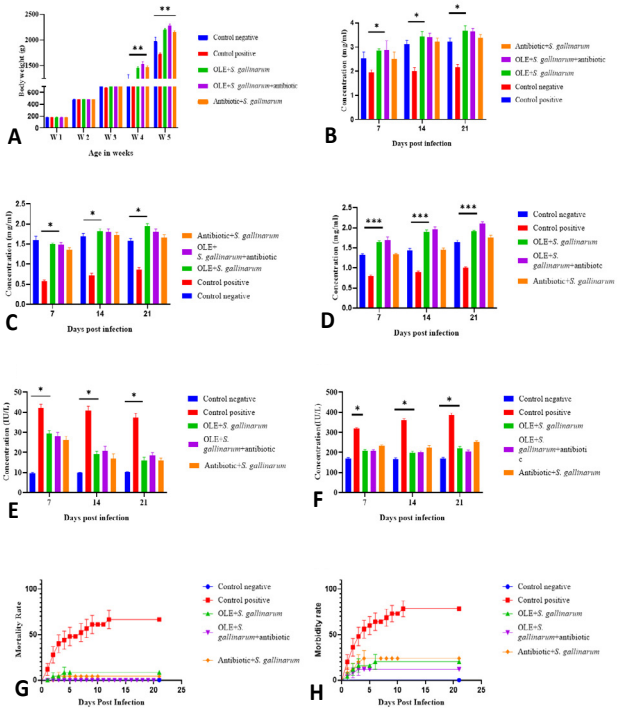

Fig. 2. Effect of different preparations of OLE on growth rate (A), total protein concentrations (B), globulin (C), albumin (D), alanine aminotransferase (E), aspartate aminotransferase (F), mortality rate (G) and morbidity rates (H) activities of normal and *Salmonella gallinarum* infected broiler chicks. For A-D, * indicates significance only with the control positive group and ** shows significance among all groups while *** means significance among the control positive and both of the OLE treated groups. For E and F, * indicates significantly higher results of control positive among all the other groups.

Present study revealed a statistically significant increase in body weight of broiler chicks supplemented with olive leaf extract and increased growth performance than that of the control group at 28th and 35th days of age (Fig. 3A). An increase in body weight of chicks due to olive leaf supplementation was found (Cayan and Erener, 2015; Oke *et al*., 2017). Previous studies revealed that the anabolic effect of olive leaf was in dose dependent manner (Shafey *et al*., 2013). In current study there was a 25% and 15% increase in body weight of the treated group with olive leave extract than that of the control positive and control negative groups of the broiler birds, respectively. There was also a significant increase in the weight gain of the chicks treated with olive leaf extract and antibiotics than that of the control group. This increase in body weight in OLE treated groups suggests its antimicrobial action against *S. gallinarum*.

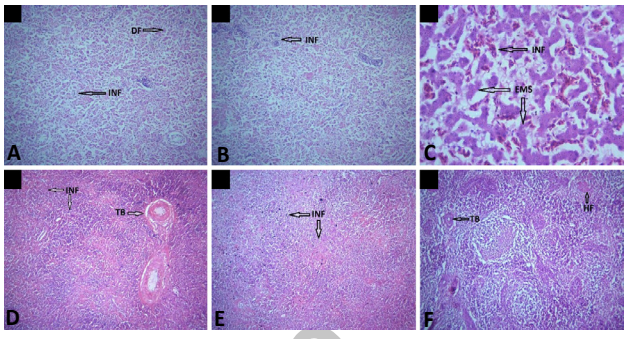

Fig. 3. Histopathological examination (A) Liver from control positive group at 21st day (B), 28th day (C) and 35th day (D) spleen from control positive group at 21st day (E) at 28th day (F) and spleen at 35th day. INF, Lymphatic infiltration; TB, thickening of blood vessels; HF, hepatic foci necrosis; DF, degenerated follicle; EMS, edematous swelling.

The total protein concentration of control positive group broiler birds was significantly lower than that of rest of the groups at 7th, 14th and 21st days post-infection (Fig. 3B). Similar trend was found in case of globulin concentration among all the groups, in OLE treated groups both of above proteins level was increased but not significantly (Fig. 3C). Albumin concentration of olive leave extract treated groups was significantly higher than that of the both of the positive and negative control groups at each slaughtering post infection (Fig. 3D), at $p \leq 0.050$ (n=7). Serum proteins like albumin globulin and total proteins are synthesized by the reticuloendothelial cells of the liver, general body tissues, spleen, and bone marrow. These proteins play a vital role in body defense by producing antibodies, maintaining oncotic pressure, acting as a transporter in the drug delivery system, and also in passive immunization (Mathew and Bhimji, 2018). The current study revealed a significant increase in the total protein, albumin, and globulin concentration in the OLE treated group than that of the control positive group; results were in line with an early study (Abdel-Maksoud *et al*., 2016). This increase in total protein indicates the protective effect of olive leaf on the liver, spleen, and bone marrow.

The activities of ALT in the control positive group were significantly higher than that of the rest of the groups at 7th, 14th and 21st days of post infection, while there was no significant difference between all other groups at each

slaughtering after the inoculation of infection (Fig. 3E) and same trend was observed in case of AST (Fig. 3F), at $p \leq 0.050$ (n=7). ALT and AST are transaminase enzymes that are considered as clinical biomarkers for the liver health. Increased concentration of these enzymes indicates damage to the liver (Hall and Cash, 2012). In the present study, the ALT and AST concentration in the serum of the OLE supplemented group was decreased significantly than that of the control groups. This decline in the concentration of ALT and AST in the Olive leaf extract supplemented groups of broiler chicks indicates the hepato-protective effect of olive leaf (Owen *et al*., 2000).

The mortality rate was found as 0, 64, 8, 0 and 4% in group A, B, C, D and E (Fig. 3G) while morbidity rate was 0, 76, 20, 12 and 24 % (Fig. 3H), respectively. The mortality rate was significantly decreased in experimental groups supplemented with OLE (10ml/l) and antibiotic (florfenicol 30mg/kg BW), both alone and in combination. Up to 80% mortality rate in brown layers due to fowl typhoid was recorded (Soufy *et al*., 2016). Decrease in mortality rate by olive leaf extract was found due to the antimicrobial activity of its phenolic contents (Elsaaed *et al*., 2014).

The primary target sites for *Salmonella* are the liver and spleen, so a histological study was performed to detect pathological changes among these organs (Fig. 3I). Hepatic tissues of infected birds were found with degenerative changes of the hepatic cord, severe hemorrhages, and edematous swelling of veins. Isolated foci of necrosis in hepatic parenchyma along with infiltration of leucocytes predominantly mononuclear cells which were mostly centered around portal triads and perivascular areas (Fig. 3A, B, C). The spleen showed congestion and thickening of blood vessels and hemorrhages, particularly below the splenic capsule, and depletion of lymphocytes along focal necrotic areas (Fig. 3D, E, F).

CONCLUSION

Current study reveals that the OLE @10ml/l DW has antimicrobial properties against *S. gallinarum.* It reduced morbidity and mortality due to *S. gallinarum* infection in broiler chicks. It has an anabolic effect on the weight gain of broiler chickens and reduces the morbidity and mortality rate. OLE also has an impact on the hematological parameters as well as serum-biochemistry of *S. gallinarum* infected broiler chicks. However, further research is required on the mechanism of action and standardization in using OLE as an antibiotic, immune stimulant, and growth promoter.

DECLARATIONS

Funding

The study received no external funding.

IRB approval

The current work was approved by ethics committee of the University of Agriculture, Faisalabad, Pakistan (DGS/39257-61) according to institutional guidelines.

Ethical statement

Animals experimental study was approved by ethics committee of University of Agriculture, Faisalabad.

Supplementary material

There is supplementary material associated with this article. Access the material online at https://dx.doi.org/10.17582/journal.pjz/20231025071251

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

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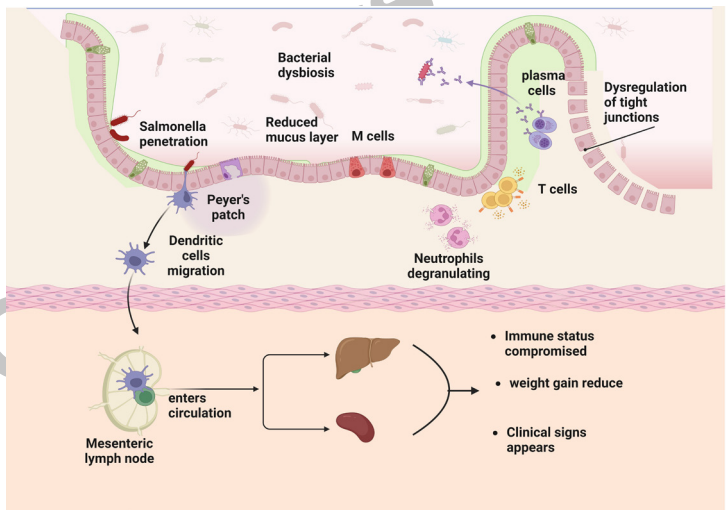
Shah Nawaz[1,2], Farzana Rizvi[1]*, Muhammad Asif[3], Muhammad Shahzad Shafiq[1], Ayesha Ramzan[1], M.Z. Shakir[1,4], Sanobar Bibi[5], Samina Shabbir[6], Ali Asif[2], Md. F. Kulyar[2]* and Zeeshan Ahmad Bhutta[7]*

[1]Department of Pathology, University of Agriculture Faisalabad, Pakistan
[2]Department of Clinical Veterinary Medicine, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[3]Department of Veterinary Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan
[4]Institute of Drug Discovery Technology, Ningbo University, China
[5]The Government Sadiq College for Women University, Bahawalpur, Pakistan
[6]Department of Chemistry, The Women University, Multan, Pakistan
[7]Laboratory of Veterinary Immunology and Biochemistry, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Republic of Korea



Supplementary Fig. 1. A brief understanding of *salmonella gallinarum* pathogenesis in chickens.

* Corresponding author: frizivi@yahoo.com, fakharealam786@hotmail.com, zee@cbnu.ac.kr
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