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Bacteriological and Clincopathological Studies on The Effect of *Olive Leaves* in Chicken infected with *Pasteurella multocida*.

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Abstract

This experiment was conducted to investigate the effects of olive leaves (OL) in chicken experimentally infected with *P. multocida* and to determine the (MIC) of (OL) water extract against *P. multocida*. A total of 60 balady chicks one-month-old (SPF) were randomly assigned to 3 groups, control and two experimental groups (20 birds / each). Chicks of the experimental groups, group (II) and (III) were intramuscularly injected with 0.2 ml/ bird of 18 hrs. broth culture of *P. multocida* containing $3x10^8$ CFU / ml. Birds of group (III) given ration containing olive leaves (5gm/kg of ration). Clinical signs, mortality rates, organ invasion, and some haemato-biochemical parameters were recorded. Blood samples were taken 3, 7 and 14 days post infection for determination of RBCs count, HB concentration, total and differential leucocytic count, serum AST, ALT, creatinine, urea, triglyceride and cholesterol levels. Results obtained showed that feeding with OL reduced signs of illness, decreased mortality rate and invasion of lung, liver and spleen with *P. multocida*, at the same time showed significant increase in RBCs count, HB concentration and significant reductions in blood levels of AST, ALT urea, and cholesterol. Also water extract of OL inhibited the growth of *P. multocida* at (MIC) 250 mg/ml. In conclusion, dietary supplementation with OL reduced bacterial invasion and improved the haemato-biochemical parameter in broiler chickens.

Introduction

Poultry production especially broilers is one of the largest and fastest growing agro-based industries in the world and provides the opportunity of meeting animal protein needs for humans. Fowl Cholera (FC) (avian cholera, avian pasteurellosis or avian hemorrhagic septicemia) is a contagious disease affecting domesticated and wild birds **(Swapnil *et al.*, 2011)**, *Pasteurella multocida* is the most common cause of fowl cholera. The incidence of fowl cholera caused by *P. multocida* is reported to be on the increase, **(Mbuthia *et al.*, 2008)** documented the occurrence of *P. multocida* among healthy-appearing family poultry in a tropical setting and concluded it to be the most common bacterial disease encountered in village chickens. Fowl cholera, caused by *P. multocida*, occurs sporadically or enzootically in most countries of the world wherever intensive poultry production occurs, and is known as a bacterial disease with major economic importance due to its high mortality **(Chrzastek, *et al.*, 2012 and Glisson, *et al.*, 2013)**. *P. multocida* causes acute septicemia and

chronic respiratory infection in birds, (**Christensen and Bisgaard 2000 and Furian, *et al.*, 2016)**.

On the other hand Olive Leaves (OL) is cheap and available plant in different seasons. It has all the same healthful qualities of olive oil without the fat and in higher concentrations. There is no basis for dosage recommendations. Many different commercial preparations of olive leaf extracts are available and vary in strength **(Mart *et al.*, 2003)**. The main medical constituents in OL are oleuropein and hydroxytyrosol, as well as other polyphenols and flavonoids **(Tripoli *et al.*, 2009)**. Oleuropein has many positive and health-promoting effects which is mostly related to its antioxidant effect that protect the body from the continuous activity of free radicals **(Al-Azzawie and Alhamdani, 2006)**. Olive leaf extract has an antimicrobial activity, it inactivate bacteria by dissolving the outer lining of microbes and it can stimulate phagocytosis **(Markin *et al.*, 2003; Owen *et al.*, 2003; Pereira *et al.*, 2007; Sudjana *et al.*, 2009 and Aytul, 2010)**. **Jemai, (2008) and Eidi *et al.*, (2009)**, recorded that, (OL) reduced blood levels of cholesterol, lipid, serum levels of glucose, uric acid, creatinine and liver enzymes. **Varmaghany *et al.*, (2013)** study the effect of dietary olive leaves supplementation for broilers at different doses (5, 10, or 15 g/kg diet) on ascites indices, hematological parameters, and broiler performance, he found that ascites-related mortality, packed cell volume, alanine aminotransferase, erythrocyte osmotic fragility, red blood cell count, and triiodothryronine level decreased linearly with increasing olive leaves supplementation.

The aim of work was: To study the antimicrobial activity of Olive leaves against *P. multocida* and to evaluate the effect of Olive leaves diet as an antibacterial agent on the blood pictures, liver function, kidney function and lipid profile in chicken experimentally infected with *P. multocida.*

Materials and Methods

Chickens: Sixty apparently healthy balady chicks one-month-old were used in this experiment, it was proved that they were free from pathogenic bacteria and parasitic infestation through cultural and serological examination (SPF).

Bacterial strain: *P. multocida* strain isolated from diseased chicken and identified by PCR in previous study was used in the experimental infection.

Olive Leaves: Olive leaves sample were rinsed thoroughly with water to remove dust and debris. Clean leaves were air dried in the shade and then grinded by a blender to fine powder, using an electric grinder and stored in an air-tight container in a dark place until extraction procedure to prevent oxidation.

Extraction procedure: According to **Cheruiyot *et al.*, (2009)** and **Masoko and Makgapeetja (2015)**: The ground leaves (1 g) were extracted with 10 mL of water by centrifugation three times with the same volume of solvent which added repeatedly . The extract was filtered, and leaved in an Avon at 50 ^{o}C for water evaporation and sterilized by filtration through 0.45 μm millipore filter and stored at 4 °C.

Agar-Well Diffusion Assay: (Andrew 2001): A concentration of 1 g/ml of the plant extracts was designed from the stock solution for agar well diffusion assay. Broth cultures of *P. multocida* strain was inoculated on the surface of Mueller Hinton agar plates by surface spreading technique. The sensitivity testing of the plant extracts was done using the agar well diffusion method. About 50 μl of the (OL) water extract, at concentrations of (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml) were dispensed into respective wells and Gentamycin (10 μg) was used as a positive control, Normal saline was used as negative control. The set up was incubated for 24 hours at 37 ^{o}C, and zones of inhibition were measured.

Bacterial isolation and identification: -Isolation and identification of *P. multocida* from experimentally infected birds was carried out according to **Collee *et al.*, (1996) and Quinn *et al.*, (2002)**.

Experimental Design

The birds were randomly divided into a control and two experimental groups (20 birds / each). The first group (I) used as control fed ration without olive leaves kept in a separate room as a control group. Group (II) received feed and water without any additive (infected group).

Group (III) received feed contain dried crushed olive leaves 5g /kg diet **(Varmaghany *et al.*, 2013),** (olive leaves treated group). At the 4th day, the experimental groups, group (II) and (III) were intramuscular infected with 0.2 ml/ birds of 18 hrs. broth culture of *P. multocida* containing $3x10^{8}$ CFU / ml (**Amany Abd-Alla 1997)**. Birds had free access to feed and water throughout the experiment. Clinical signs, mortality rate, organs invasion, hematological changes and serum biochemical changes were recorded.

Diagnostic kits: Commercial diagnostic kits were purchased from Sinreact, Diamond, Egypt and Biodiagnostic for determination of hemoglobin (Hb), aspartate aminotranseferase (AST), alanine aminotransferase (ALT), Creatinine, urea, Cholesterol and Triglyceride.

Samples

To determine organs invasion, after challenge, samples of lung, liver and spleen were taken from birds of each experimental group. Samples were appropriately removed, and after recording, lung, liver and spleen weights,

organs were homogenized separately. The homogenates of each organ were diluted 1:10 with a sterile solution of 0.1% peptone water (Oxoid) and 100 µL was spread on tryptic soy agar, supplemented with 5% sheep blood, and incubated at 37^0C for 24- 48hs. In cases in which growth was detected, a series of biochemical confirmatory tests were performed according to **Collee *et al.*, (1996)** and **Quinn *et al.*, (2002)**. Organ invasion was evaluated by enumeration the number of CFU of *P. multocida* in lung, liver and spleen samples. Also mortality rate for each group was recorded.

-Blood samples: Blood samples were collected from the wing vein after three days, one week and two weeks post infection. Blood samples were divided into 2 parts; the first part was collected on EDTA for detection of erythrocytes (RBCs) count, Hb concentration, total and differential leucocytic count, according to **(Feildman *et al.*, 2000)**. The second part was collected into plain centrifuge tube for serum separation and determination of aspartate amino transeferase AST and alanin aminotransferase ALT activities according to **(Reitman and Frankel, 1957)**, Creatinine according to **(Young *et al.*, 1975)**, urea according to **(Patton and Crouch 1977)**, cholesterol according to **(Henry *et al.*, 1974)** and triglyceride according to **Scheettler and Nussel (1975)**

Statistical analysis:-Collected data from the different groups of chickens were statistically analyzed for the mean and standard error using GraphPad InStat program version 3.

Results and Discussion

Clinical signs: - Experimentally Infected birds showed depression, dyspnea, ruff feather and diarrhea (fig1) reduced signs of illness were observed in birds given olive leaves.

P. multocida is an animal pathogen of worldwide economic significance that causes fowl cholera in poultry and wild birds **{Kangpeng *et al.*, (2016) and Furian *et al.*, (2016)}**. In the present study the birds exhibited signs of respiratory distress within few days of giving infection with *P. multocida*, **Christensen and Bisgaard, (2000)** showed that *P. multocida* can cause peracute, acute and chronic infections which could be associated with high mortalities, regarding to tables (1) it was clear that experimental infection with *P. multocida* induced low mortality rate in olive leaves treated group, **Varmaghany *et al.*, (2013)** and **Eiman *et al.*, (2014)** recorded that mortality due to *E. coli* infection or ascites was decreased with olive leaf supplementation.

Glisson *et al.*, (2013) stated that *P. multocida* was readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, from table (2) invasion of lung, liver and spleen with *P. multocida* was

decreased by addition of olive leave in ration of infected chicken, this result may be due to the antibacterial action of (OL) and ⁄ or immune-stimulant activities of (OL), **Cheruiyot, *et al.*, (2009), Morteza, *et al.*, (2012) Mahmoud, *et al.*, (2013) and Masoko and Makgapeetja (2015)** indicated that (OL) extracts exhibited a significant bactericidal activities against many of Gram negative and Gram positive microorganisms and using of olive leaves had the beneficial effect in controlling the microbial infections.

a- **Hemaogrm**: .Results of the changes in hemogram in chicken of the experimental groups are shown in table (3). It revealed a significant decreased of RBCs count in (group II) 3 and 7 days post infection, while (group III) showed significant increase in RBCs count and HB concentration 3 days post infection. Leukogram showed significant increase in leukocyte in (group III) 14 days post infection while non-significant increase in leukocyte count in (group II) 7 days post infection. Lymphocytosis was observed in (group III) 7 and 14 days post infection. Significant increase in monocytes was observed in (group II) and (group III) 3 days post infection.

Haematological changes are commonly used to determine the body status and to assess the impact of environmental, nutritional and or pathological stress **(Elagib and Ahmed, 2011)**. Reduction in erythrocytic count in (group II) is a sign of anemia. The occurrence of anemia in fowl cholera has been noted by earlier workers although whether haemolytic or otherwise was not stated **(Swapnil *et al.*, 2011)**, probably, the cause of anemia in the present study is due to the bacterial septicemia. On the other hand the olive leaves treatment enhances the erythropoiesis as shown by the significant increase in the RBCs and Hb concentration in the groups III as compared with the control. This improvement in erythropoiesis may be related to the enhancement of antioxidant activity of olive leaves in RBCs **(Al-Azaria and Alhamdani, 2006)**. Our result revealed increase values of WBCs in (group II) 7days post infection was agree with **(Mahmood *et al.*, 2004, Islam *et al.*, 2004 and Swapni *et al.*, 2011),** the endotoxin, a cell wall component of *P. multocida* might be responsible for the alterations of circulating leukocyte counts and its structure. Leukocytosis with lymphocytosis in (group III) may indicate an immune-stimulatory effect of olive leaves **(Mahmoud *et al.*, 2013 and Eiman *et al.*, 2014)**.

b- **Serum biochemistry:** Results of the changes in serum biochemical parameter in chicken of the experimental groups are shown in Table (4). Serum Aspartate aminotransferase (AST) concentration was increased in birds of (group II) 14 days post infection. Determination of urea showed significant increase on the

7th and 4th days post infection in (group II). Levels of (ALT), (AST) and urea in serum of chicken in group III were comparable to the control group .Triglyceride was increased significantly in (group II) 7days post infection. Significant reductions in blood levels of cholesterol was observed in (group III) 14 days post infection.

Table (1) showing that mortality rate in (G. III) chicken infected with P. multocida and feed on ration containing (OL) was 5 % while in (G. II) infected with P. multocida and not feed on ration containing (OL) it reached to 20 %**.**

Table (2) clearing that invasion of lung, liver and spleen with *P. multocida* was higher in non-treated group (G II) at all three intervals.

The increased value in AST in (group II) could be due to the hepatopathy caused by *P. multocida*, it is well documented, that elevation in concentration of plasma enzymes occur as a result of their escape from disrupted hepatic parenchyma cells or altered membrane permeability, similar to present observations **(Shivachandra *et al.*, 2005)** reported mild to moderate congestion of blood vessels and haemorrhages in the intermycium of the heart, lung and liver in cases of fowl cholera. The present study revealed that the birds given olive leaves showed improvement in the activity of serum enzyme AST and ALT in group III, this could be due to the antioxidant and anti-inflammatory properties of olive leaves **(Visioli and Galli 2002 and Abdel-Hamid *et al.*, 2011).**

Elevations of serum urea concentration in (group II) was suggested as a significant functional impairment of kidney caused by *P. multocida*. This results is in agreement with **(Amany Abd-Alla 1997).** The present study showed amelioration of serum urea concentration in (group III) received OL these may be related to the antioxidant property of olive leaves. These results are in accordance with **(Tavafi *et al.*, 2012 and Mahmoud *et al.*, 2013).**

Reductions in blood levels of cholesterol in group III could be due to the hypocholesterolemic property of olive leaves **(Fki *et al.*, 2005, Jemai, 2008, and Mahmoud *et al.*, 2013)** recorded that olive leaves reduce the intestinal absorption of cholesterol or decrease its synthesis by liver **(Krzeminski, 2003)**. Also olive leaves stimulates the biliary secretion of cholesterol and its excretion in the feces **Siamak *et al.*, 2014).**

From table (5) The Minimal Inhibitory Concentrations of (OL) water extract against *P. multocida* was 250 mg/ml

Regarding to table (5) water extract of (OL) exhibited bactericidal activities against *P. multocida* with (MIC) 250 mg/ml, **Masoko and Makgapeetja (2015)** recorded that water extract of (OL) exhibited bactericidal activities against some Gram negative and Gram positive bacteria with (MIC) average 208 mg/ml, while **Mukadderat, *et al.*, (2014)** showed that (MIC) of (OL) extract against some Gram negative and Gram positive bacteria ranged from

(160 to 320) mg/ml. Antimicrobial activities, of olive leave extract were reported by **(Visioli *et al.*, 2002; Owen *et al.*, 2003; Micol *et al.*, 2005** and **Sanchez *et al.*, 2007).** In conclusion, olive leaves supplementation had positive effects on hemogrm, liver enzymes, kidney function, blood lipids, reduce mortality, organ invasion and the severity of *P. multocida* infection in birds.

Fig (1) *P. multocida* experimentally infected birds showing depression, dyspnea, ruff feather and diarrhea.

Table (1): Mortality rate of groups under experiment.

Group	N. of birds	N .of died birds	Mortality rate
I – Negative control	20	0	0 %
II-*P. multocida* without olive leaves	20	4	20 %
III -*P. multocida* + olive leaves	20	1	5 %

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Table (2): Invasion of different organs at d 3, 7 and 14 days after inoculation with *P. multocida*

Time	Group	Lung	Liver	Spleen
3days post infection	I	0	0	0
	II	2.0×10^5	1.3×10^5	1.5×10^5
	III	1.2×10^5	1.0×10^5	0.8×10^5
7days post infection	I	0	0	0
	II	1.6×10^5	1.1×10^5	1.0×10^5
	III	1.0×10^5	0.7×10^5	0.5×10^5
14days post infection	I	0	0	0
	II	1.5×10^5	1.0×10^5	0.8×10^5
	III	0.3×10^5	0	0

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Table (3): mean values ± S.E. of hemogram in different experimental group of chickens.

Time	Group	RBCs x10^6/ul	HB g/dl	WBCs x10^3/ul	Differential leucocytic count %		
					Lymphocyte	Neutrophil	Monocyte
3days post infection	I	2.86 ± 0.16	12.22 ± 0.12	26.00 ± 1.14	81.40 ± 1.96	14.00 ± 1.08	3.00 ± 0.66
	II	2.10 ± 0.15*	11.89 ± 0.16	23.80 ± 1.59	79.75 ± 3.11	11.00 ± 0.57	6.75 ± 0.25*
	III	3.82 ± 0.36*	13.85 ± 0.25*	29.80 ± 2.70	80.25 ± 1.37	13.66 ± 0.33	6.75 ± 0.25*
7days post infection	I	2.93 ± 0.18	12.22 ± 0.18	25.00 ± 0.70	81.40 ± 1.96	15.00 ±0.57	3.00 ± 0.54
	II	2.03 ± 0.08*	10.41 ± 0.08	30.33 ± 3.71	80.00 ± 1.95	15.00 ± 2.00	3.33 ± 0.88
	III	2.25 ± 0.12	10.18 ± 0.12	20.90 ± 2.30	89.00 ± 0.70*	9.33 ± 1.20	4.00 ± 0.57
14days post infection	I	2.96 ± 0.15	12.27 ± 0.16	26.00 ± 1.14	81.40 ± 1.96	15.00 ± 0.75	3.00 ± 0.54
	II	2.70 ± 0.14	10.41 ± 0.15	23.00 ± 1.64	80.00 ± 1.95	15.75 ± 1.25	4.25 ± 0.85
	III	3.14 ± 0.08	14.200 ± 0.57	31.00 ± 3.61*	89.00 ± 0.70*	9.33 ± 0.33	2.50 ± 0.28

Group I Normal control group - Group II Infected group - Group III Olive leaves supplemented group, *Significantly different from normal control group, $P < 0.0$ **Highly significantly different from normal control group, $P < 0.001$

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Table (4): mean values ± S.E. of some serum biochemical parameters in different experimental group of chickens.

Time	Group	AST U/ml	ALT U/ml	Creatinine mg/dl	Urea mg/dl	Triglyceride mg/dl	Cholesterol mg/dl
3days post infection	I	118.00± 8.00	9.00 ± 0.91	0.52 ± 0.02	13.83±0.46	38.75± 6.60	110.00± 7.02
	II	154.00± 21.06	10.40 ± 1.53	0.60 ± 0.02	15.02±0.99	41.00 ± 11.36	144.20 ± 19.08
	III	127.00 ± 10.53	9.20 ± 1.15	0.55 ± 0.02	14.44±0.30	40.60 ± 4.27	130.33± 25.62
7days post infection	I	114.33 ± 5.89	9.00 ±0.91	0.52 ± 0.02	13.833±0.46	38.75± 6.60	110.00. ±7.02
	II	97.33 ± 4.84	8.00 ± 0.57	0.6 2± 0.03	15.16±0.29*	73.66 ± 4.48 *	108.67 ±5.81
	III	122.50 ± 10.50	8.400 ± 0.74	0.54 ± 0.01	13.70±0.05	36.66 ± 3.66	94.33 ±9.24
14days post infection	I	116.33-± 4.91	8.33± 0.88	0.50 ± 0.01	13.45±0.25	38.75 ±6.60	110.00 ± 7.02
	II	169.33± 13.86*	9.50 ± 1.5	0.52 ± 0.01	16.45±1.65*	61.33±3.75	148.00± 21.22
	III	120.33± 2.33	7.00 ± 0.57	0.50 ± 0.02	14.10±0.30	41.500 ± 0.50	79.50± 11.60*

Group I Normal control group - Group II Infected group - Group III Olive leaves supplemented group *Significantly different from normal control group, $P < 0.05$, **Highly significantly different from normal control group, $P < 0.001$

Table (5) Minimal Inhibitory Concentrations of (OL) water extract against *P. multocida*

MIC (mg/ml)	**500**	**250**	**125**	**62.5**	**31.25**
Zone of inhibition (mm)	**17**	**10**	**-**	**-**	**-**

Gentamycin (10 μg) (bioMerieux) – Inhibitory zone (13- 15 mm)

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