

## CLINICOPATHOLOGICAL AND IMMUNOLOGICAL EFFECTS OF USING FORMALIZED KILLED VACCINE ALONE OR IN COMBINATION WITH PROPOLIS AGAINST *Pasteurella multocida* CHALLENGE IN RABBITS

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**Abstract:** Pasteurellosis is a common and troublesome issue of rabbits causing serious disorders. The immunization procedures are constantly the greatest preventive measures. In the present study, 40 New Zealand rabbits were used to investigate the protective efficacy of formalized *Pasteurella multocida* vaccine alone or in combination with propolis. The animals were divided into four equal groups (I-IV); negative control group, challenged non-vaccinated group, vaccinated challenged group and vaccinated propolis administered challenged group respectively. At the end of the 2<sup>nd</sup> and 6<sup>th</sup> weeks of the experiment, blood samples were collected from ear vein of rabbits for hematological, plasma, and serum examinations. The rabbits were then anaesthetized and sacrificed to collect tissue specimens from liver, kidneys, spleen and lungs for histopathological study. The results showed that using of propolis in combination with killed vaccine of *Pasteurella multocida* improved the immune response by increasing the leukocyte phagocytic activity against *Pasteurella multocida* (from 23.80% to 60.80%). Moreover, the clinicopathological findings including hemogram (RBCs count, Hb content, PCV, RBCs indices, platelets, total and differential leukocytes count), and hepato-renal function tests (ALT, AST, ALP, bilirubin, urea and creatinine), as well as, histopathological findings were better in infected rabbits treated with propolis- killed vaccine than using killed vaccine alone.

**Key words:** propolis; pasteurellosis; phagocytosis; nitric oxide; *P. formalized killed vaccine*

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### Introduction

One of the common diseases that affect rabbits' generation is pasteurellosis (1). Pasteurellosis affects rabbits of 4– 8 weeks old causing manifestations ranging from lethal septicemia, serious pleuritis, and pneumonia to less serious condition as multiple abscesses, rhinitis, and otitis media. The utilization of antibiotics has been in part effective in controll-

ing pasteurellosis in rabbits, since they don't totally dispose of the bacterium (2). Control of pasteurellosis in rabbits is achieved by vaccination against *Pasteurella multocida* infection. Vaccination of rabbits with killed vaccine (bacterin) frequently brings about insufficient protection in the field when used in a single dose (3).

Immunization studies on rabbits' pasteurellosis have been accounted for the utilization

of inactivated forms as formalized (4), joined with oil adjuvant (5) or heat treated (6). Propolis (honey bee stick) is a resinous hive item, created by bumble bees from different plant sources. It has a few natural properties as antimicrobial, anti-inflammatory and immunomodulatory (7-9). Propolis has a several clinicopathological impacts on rebuilding of renal and hepatic functions (10). The propolis extract may have gone about as a right hand adjuvant substance, potentiating the humoral response initiated by the antigen (11). Thus, the aim of the present work was to study the effect of formalized killed *P. multocida* vaccine either injected alone or in combination with propolis as an adjuvant against experimental challenge of rabbits with *P. multocida* strain based on selective hematological (RBCs, Hb, PCV, MCV, MCHC, TLC, neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets), biochemical (ALT, AST, ALP, bilirubin, urea, creatinine and nitric oxide), immunological (phagocytic percent) and histopathological (liver, kidneys, spleen and lungs) investigations.

## Material and methods

### *Animals*

A total of 40 apparently healthy white New Zealand rabbits of 1 kg average body weight and 6–8 weeks old were obtained from laboratory animal house, Faculty of Veterinary Medicine, Zagazig University. All rabbits were not previously vaccinated against pasteurellosis and with no history of pasteurellosis. They were kept under hygienic conditions, housed in metal cages at Experimental Animal Unit, Faculty of veterinary Medicine, Zagazig University and fed on balanced ration and water *ad-libitum* and maintained in a 12 h light-dark cycle at a controlled temperature (21–24°C) and humidity (50–60%). They were kept for 15 days without medication for acclimatization before beginning the study. The care and welfare of animals conformed to the guidelines of the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

### *The used vaccine, propolis, challenging bacteria and chemicals*

The formalized killed vaccine for *P. multocida* (9-10<sup>9</sup> bacterial cell/ml- batch number: 56) that was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Propolis that was obtained from bee hives located in Sharkia Governorate, Egypt. Propolis bulk was cut into small pieces, mixed with deionized water and then shook at 95°C for 2 h to prepare the therapeutic dose (50 mg propolis in each 1 ml). Then it was cooled to room temperature and centrifuged at 1500 rpm for 5 min to obtain the supernatant (12).

### *Challenging bacteria*

The strain of *P. multocida* was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, as lyophilized ampoules (1 ml containing 5×10<sup>9</sup> bacterial cell of *P. multocida* serotype A). It was activated by culturing in nutrient broth followed by intraperitoneal inoculation (I/P) in Swiss mice, and then re-isolated from heart blood of mice on nutrient agar plates (Difco). Bacterial colonies were suspended in sterile saline, and the density was adjusted to contain 5×10<sup>9</sup> bacterial cell/ml. The suspension was used for S/C injection of rabbits in the challenge test (13). All chemicals and stains were of analytical grade and obtained from Sigma Co., and El Gomhoria Co., Egypt.

### *Experiment design*

Forty New Zealand rabbits were divided into 4 groups (I-IV), each group contained 10 rabbits as following; group (I): non vaccinated and non challenged control group, group (II): non-vaccinated challenged group and group (III): the vaccinated challenged group in which rabbits were injected S/C with single dose of (1 ml/kg BW) formalized killed *P. multocida* vaccine according to Okerman and Spanoghe (3) and Osama (14), group (IV): the vaccinated propolis administered group in which rabbits were injected S/C with *P. multocida* vaccine (a single S/C dose of 1 ml/kg BW) mixed with propolis (50 mg /kg BW) as an adjuvant according to Nassar et al. (15). At the end of the

5<sup>th</sup> week from starting the experiment, rabbits in groups II, III and IV were challenged S/C with broth culture of virulent *P. multocida* strain (0.2 ml/ kg BW) (15).

#### *Sampling*

Blood samples were collected from the marginal ear vein of rabbits in all groups at the end of 2<sup>nd</sup> and 6<sup>th</sup> weeks of the experiment. They were divided into three parts. The 1<sup>st</sup> part (1ml rabbit blood) was placed in clean Wasserman tubes containing disodium salts of EDTA for hematological examination. The 2<sup>nd</sup> part (5ml rabbit blood) was placed in a chemical free test tube without anticoagulant to separate a clear serum for biochemical analysis (16). The 3<sup>rd</sup> part (3ml) was collected in heparinized tubes for phagocytic activity. Tissue specimens from liver, kidneys, spleen and lungs from all experimental groups were collected at the end of the 6<sup>th</sup> week of the experiment for histopathological examination.

#### *Hematological studies*

Complete blood picture was carried out using an automated hematology analyzer, Sysmex IV2000 (hematology analyzer), UK. This was done at Veterinary Animal Reseach, Sharkia.

#### *Clinicobiochemical analysis*

All biochemical tests were performed using colorimetric kits (BioMerieux, ABC Diagnostics, Vitro Scient, Quimica Clinica Aplicada S.A. (QCA), Spinreact, ELITech and Diamond-Diagnostics) according to the manufacturer's instruction. The applied biochemical tests were measured the serum levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) according to the method of Reitman and Frankel (17), Alkaline Phosphatase (ALP) according to modified method of Tietz (18), Bilirubin levels (total and direct) according to Fuehr (19), serum creatinine and urea according to the method of Henry (20) and Fawcett and Scott (21), respectively. Nitric oxide based on Griess reaction as cited in Montgomery and Dymock (22). Indirect bilirubin was calculated by

subtracting the direct bilirubin level from the total bilirubin level obtained.

#### *Phagocytic activity*

Heat-inactivated *Candida glabrata* (*C. glabrata*) was used as a microbial model to evaluate the phagocytic activity of the rabbit monocytes. In brief, leukocytes were separated from the heparinized blood samples (23,24). One ml of the adjusted viable leukocytes suspension (leukocytes in RPMI 1640 with 5% of pooled rabbit serum) was placed in a sterile plastic tube, to which 1 ml of the prepared heat inactivated *Candida glabrata* was added. The tubes were then incubated at 27°C for 30 min in a humidified 5% CO<sub>2</sub> incubator. Then the tubes were centrifuged at 2500 rpm for 5 min, and the supernatant was removed with Pasteur pipette leaving a small part into which the sediment was re-suspended. Slide smears were prepared from the deposit, air dried and then stained with Leishman's stain. Under a light microscope using oil immersion lens, phagocytic cells were counted randomly in about ten microscopic fields. The number of phagocytic cells with engulfed yeast was recorded. The phagocytic activity was evaluated according to the following equation (25):

Phagocytic percentage (**P %**) =

$$\frac{\text{Number of phagocytes with engulfed yeast cells}}{\text{Total number of phagocytes}} \times 100$$

#### *Histopathological examination*

Specimens from the liver, kidneys, spleen and lungs from all groups were collected at the end of 6<sup>th</sup> week post-treatment, and then fixed in 10% neutral buffered formalin for 48h, then washed overnight under running water. The washed specimens were dehydrated by using up graded concentrations of ethyl alcohol starting with 75% and ending with absolute alcohol, cleaned in xylene two times, each for 2 hrs. There after the specimens were placed in crucible containing soft paraffin and kept in an oven at 60°C for 12h. Paraffin sections of 5 microns thickness were prepared and stained

with H & E stains for histopathological examination (26).

#### *Statistical analysis*

The obtained data was analyzed using one way ANOVA (SPSS Inc. Released 2007. SPSS for windows, version 16.0. Chicago, SPSS Inc.) (27). Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a ( $p = 0.5$ ).

## **Results**

### *Hematological findings*

Regarding the erythrogram, as shown in Table (1) comparing with the control non-infected group (I), the non vaccinated challenged group (II) showed non-significant changes at the values of RBCs ( $5.68 \pm 0.07$ ), Hb ( $11.38 \pm 0.10$ ), PCV ( $35.71 \pm 0.90$ ), MCV ( $62.89 \pm 1.44$ ), MCHC ( $31.94 \pm 0.67$ ), and platelets ( $456.8 \pm 13.58$ ) at the end of the 2<sup>nd</sup> week from starting the experiment. Meanwhile, at the end of the 6<sup>th</sup> week, it showed macrocytic hypochromic anemia with thrombocytopenia. On the other hand, the vaccinated challenged rabbits of group (III) showed normocytic normochromic anemia at the end of the 2<sup>nd</sup> week with non-significant changes at the counts of platelets ( $448.8 \pm 36.12$ ) but at the end of the 6<sup>th</sup> week, they showed non-significant changes at the erythrogram indices with non-significant changes at platelets count also. On the other hand, vaccinated propolis administered challenged group (IV) did not show any significant changes in the erythrogram indices and platelets count during all the experimental periods.

### *Leukogram and Immunological results*

At the end of the 2<sup>nd</sup> week of the experiment compared to the control group as illustrated in Table (2), the infected non-vaccinated group (II) showed a non significant change in the counts of total leukocytes ( $8.84 \pm 0.42$ ) including neutrophils ( $3.84 \pm 0.28$ ), lymphocytes ( $4.21 \pm 0.28$ ), monocytes ( $0.77 \pm 0.09$ ), eosinophils ( $0.08 \pm 0.02$ ) and basophils ( $0.24 \pm 0.09$ ) counts. Meanwhile, at the end of

the 6<sup>th</sup> week, group (II) showed a highly significant increase in total leukocytic ( $13.35 \pm 0.20$ ) and neutrophilic ( $8.01 \pm 0.25$ ) count with non-significant changes at the other leukocytes. On the other side, group (III) revealed leukocytosis ( $11.63 \pm 0.39$ ) and lymphocytosis ( $5.99 \pm 0.26$ ) at the end of the 2<sup>nd</sup> week with non significant change in leukogram variables at the end of 6<sup>th</sup> week of the experiment.

Group (IV) showed leukocytosis, neutrophilia, lymphocytosis and monocytosis with non significant change in eosinophils and basophils along the experimental periods. The phagocytic percent of group (II) (Figure 1b) was non-significantly changed ( $25.6 \pm 1.5$  and  $23.8 \pm 1.24$ ) than the control group (I) along experimental periods (Figure 1a), elevated in group (III) ( $35.8 \pm 1.24$ ) (Figure 1c,d) and highly increased in group (IV) ( $60.20 \pm 1.59$  and  $60.8 \pm 1.74$ ) (Figure 1e,f).

### *Biochemical results*

At the 2<sup>nd</sup> weeks of the experiment as shown in Table (3), group (II) showed non significant changes in serum ALT ( $16.5 \pm 0.39$ ), AST ( $15.2 \pm 0.41$ ), ALP ( $40.12 \pm 0.94$ ), total ( $0.94 \pm 0.06$ ), direct ( $0.24 \pm 0.02$ ), and indirect bilirubin ( $0.71 \pm 0.07$ ), urea ( $34.74 \pm 1.05$ ), creatinine ( $0.96 \pm 0.05$ ) and nitric oxide ( $0.7 \pm 0.12$ ) but these parameters were increased at the 6<sup>th</sup> week. However, group (III) at the 2<sup>nd</sup> week showed a significant increase in serum ALT ( $18.32 \pm 0.85$ ), AST ( $19.58 \pm 0.38$ ), total ( $1.36 \pm 0.06$ ), indirect bilirubin ( $1.1 \pm 0.08$ ) and nitric oxide ( $0.78 \pm 0.07$ ) with non significant changes in the serum ALP ( $44.82 \pm 1.59$ ), direct bilirubin ( $0.25 \pm 0.02$ ), urea ( $33.79 \pm 1.33$ ) and creatinine ( $0.98 \pm 0.03$ ). However, at the 6<sup>th</sup> week, it showed marked decrease in the serum activity of ALT ( $24.49 \pm 0.53$ ), AST ( $22.37 \pm 1.04$ ), ALP ( $44.31 \pm 1.21$ ), total ( $1.61 \pm 0.06$ ), indirect bilirubin ( $1.32 \pm 0.05$ ), urea ( $34.83 \pm 1.05$ ), creatinine ( $0.98 \pm 0.05$ ) and nitric oxide ( $2.06 \pm 0.05$ ) with non-significant changes at serum level of direct bilirubin ( $0.29 \pm 0.01$ ).

On the other hand, group (IV) at the 2<sup>nd</sup> week did not show any significant changes in all previously mentioned parameters comparing

with control group (I), but at the 6<sup>th</sup> week, it showed a decrease in urea ( $22.0\pm 0.95$ ), creatinine ( $0.6\pm 0.03$ ) and direct bilirubin ( $0.22\pm 0.01$ ) levels with non significant changes in other parameters. However, group (IV) showed good improvement of these parameters when compared with group (II).

#### *Histopathological alterations*

In group (II), Liver of rabbit showed multiple focal areas of coagulative necrosis surrounded by line of demarcation (Figure 2A). Furthermore, vascular congestion and mononuclear cell infiltration with widening in the glomerular space of kidney were recorded (Figure 2D). Meanwhile, spleen showed marked polymorphonuclear cell infiltration (Figure 2G). Lung also showed extensive hemorrhage (presence of large numbers of extravasated RBCs in the alveolar lumens)

(Figure 2J). The vaccinated challenged group (III) showed mononuclear cell infiltration in the portal areas of liver (Figure 2B), vascular congestion, with perivascular edema and mononuclear cell infiltration (Figure 2E) of kidney, marked thickening of the splenic capsule of spleen (Figure 2H), pneumonic area (represented by marked vascular congestion, and leukocytic infiltration) of lung (Figure 2K). The vaccinated propolis administered challenged group (IV) showed normal hepatocytes with mild portal congestion of liver (Figure 2C), normal renal epithelium except for very mild vacuolations (Figure 2F) of kidney, normal red and white pulps except for mild hemosiderosis of spleen (Figure 2I), normal pulmonary tissue except for a focus having mild congestion and few mononuclear cell infiltration of lung (Fig. 2L).

**Table 1:** Erythrogram and platelets count (mean values $\pm$ SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2<sup>nd</sup> and 6<sup>th</sup> weeks of the experiment

	RBCs ( $\times 10^6/\mu\text{l}$ )	Hb (g/dl)	PCV %	MCV (fl)	MCHC%	Platelets ( $\times 10^3/\mu\text{l}$ )
<b>2<sup>nd</sup> week</b>						
<b>Control (GI)</b>	5.63 $\pm$ 0.14 <sup>a</sup>	11.52 $\pm$ 0.31 <sup>a</sup>	37.46 $\pm$ 1.00 <sup>a</sup>	66.58 $\pm$ 1.82 <sup>abc</sup>	30.77 $\pm$ 0.55	483.60 $\pm$ 57.53
<b>Non-vaccinated Challenged (GII)</b>	5.68 $\pm$ 0.07 <sup>a</sup>	11.38 $\pm$ 0.10 <sup>a</sup>	35.71 $\pm$ 0.9 <sup>ab</sup>	62.89 $\pm$ 1.44 <sup>c</sup>	31.94 $\pm$ 0.67	456.80 $\pm$ 13.58
<b>Vaccinated Challenged (GIII)</b>	5.08 $\pm$ 0.11 <sup>b</sup>	9.94 $\pm$ 0.10 <sup>b</sup>	33.24 $\pm$ 1.51 <sup>b</sup>	65.29 $\pm$ 1.77 <sup>bc</sup>	30.11 $\pm$ 1.15	448.80 $\pm$ 36.12
<b>Vaccinated Propolis administered (Challenged GIV)</b>	5.69 $\pm$ 0.03 <sup>a</sup>	11.86 $\pm$ 0.20 <sup>a</sup>	36.64 $\pm$ 0.74 <sup>ab</sup>	64.4 $\pm$ 1.20 <sup>bc</sup>	32.39 $\pm$ 0.37	437.80 $\pm$ 30.05
<b>6<sup>th</sup> week</b>						
<b>Control (GI)</b>	5.60 $\pm$ 0.09 <sup>a</sup>	11.34 $\pm$ 0.20 <sup>a</sup>	34.30 $\pm$ 0.65 <sup>a</sup>	61.33 $\pm$ 1.78 <sup>b</sup>	33.11 $\pm$ 0.90 <sup>a</sup>	469.00 $\pm$ 48.10 <sup>a</sup>
<b>Non-vaccinated Challenged (GII)</b>	3.34 $\pm$ 0.14 <sup>b</sup>	8.46 $\pm$ 0.16 <sup>b</sup>	31.10 $\pm$ 0.44 <sup>b</sup>	102.43 $\pm$ 6.87 <sup>a</sup>	25.11 $\pm$ 0.96 <sup>b</sup>	213.20 $\pm$ 46.43 <sup>b</sup>
<b>Vaccinated Challenged (GIII)</b>	5.16 $\pm$ 0.26 <sup>a</sup>	10.90 $\pm$ 0.18 <sup>a</sup>	33.86 $\pm$ 1.19 <sup>ab</sup>	60.76 $\pm$ 2.51 <sup>b</sup>	32.54 $\pm$ 0.32 <sup>a</sup>	428.20 $\pm$ 22.19 <sup>a</sup>
<b>Vaccinated Propolis administered (Challenged GIV)</b>	5.19 $\pm$ 0.16 <sup>a</sup>	10.84 $\pm$ 0.19 <sup>a</sup>	33.13 $\pm$ 1.04 <sup>ab</sup>	63.82 $\pm$ 1.16 <sup>b</sup>	31.29 $\pm$ 0.73 <sup>a</sup>	441.40 $\pm$ 12.15 <sup>a</sup>

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a.

**Table 2:** Leukogram ( $\times 10^3/\mu\text{l}$ ) and phagocytic percentage (mean $\pm$ SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2<sup>nd</sup> and 6<sup>th</sup> weeks of the experiment

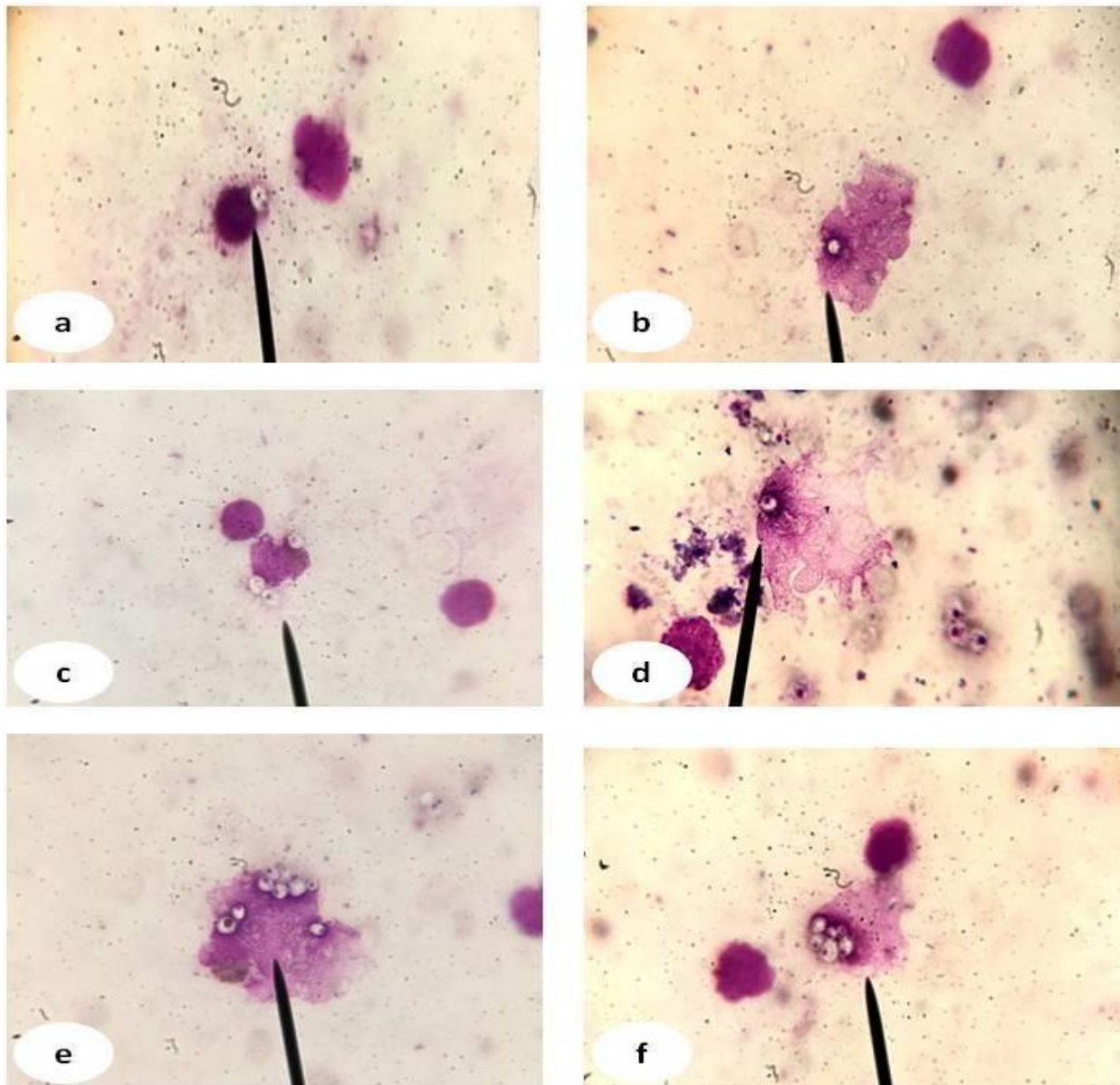
	TLC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Phagocytic %
<b>2<sup>nd</sup> week</b>							
<b>Control (GI)</b>	9.16 $\pm$ 0.74 <sup>c</sup>	4.11 $\pm$ 0.15 <sup>bc</sup>	3.98 $\pm$ 0.47 <sup>b</sup>	0.78 $\pm$ 0.28 <sup>b</sup>	0.06 $\pm$ 0.01	0.23 $\pm$ 0.06	26.20 $\pm$ 1.39 <sup>c</sup>
<b>Non-vaccinated Challenged (GII)</b>	8.84 $\pm$ 0.42 <sup>c</sup>	3.84 $\pm$ 0.28 <sup>c</sup>	4.21 $\pm$ 0.28 <sup>b</sup>	.077 $\pm$ 0.09 <sup>b</sup>	0.08 $\pm$ 0.02	0.24 $\pm$ 0.09	25.60 $\pm$ 1.50 <sup>c</sup>
<b>Vaccinated Challenged (GIII)</b>	11.63 $\pm$ 0.39 <sup>b</sup>	4.53 $\pm$ 0.18 <sup>b</sup>	5.99 $\pm$ 0.26 <sup>a</sup>	0.81 $\pm$ 0.07 <sup>b</sup>	0.09 $\pm$ 0.01	0.20 $\pm$ 0.08	35.80 $\pm$ 1.24 <sup>b</sup>
<b>Vaccinated Propolis administered (Challenged GIV)</b>	14.41 $\pm$ 0.38 <sup>a</sup>	6.44 $\pm$ 0.17 <sup>a</sup>	6.03 $\pm$ 0.26 <sup>a</sup>	1.63 $\pm$ 0.18 <sup>a</sup>	0.08 $\pm$ 0.02	0.24 $\pm$ 0.06	60.20 $\pm$ 1.59 <sup>a</sup>
<b>6<sup>th</sup> week</b>							
<b>Control (GI)</b>	9.29 $\pm$ 0.41 <sup>b</sup>	4.40 $\pm$ 0.19 <sup>c</sup>	4.03 $\pm$ 0.60 <sup>b</sup>	0.59 $\pm$ 0.04 <sup>bc</sup>	0.06 $\pm$ 0.01	0.21 $\pm$ 0.08	25.60 $\pm$ 1.50 <sup>c</sup>
<b>Non-vaccinated Challenged (GII)</b>	13.35 $\pm$ 0.20 <sup>a</sup>	8.01 $\pm$ 0.25 <sup>a</sup>	4.50 $\pm$ 0.15 <sup>b</sup>	0.63 $\pm$ 0.04 <sup>b</sup>	0.05 $\pm$ 0.00	.020 $\pm$ 0.05	23.80 $\pm$ 1.24 <sup>c</sup>
<b>Vaccinated Challenged (GIII)</b>	10.21 $\pm$ 0.71 <sup>b</sup>	4.24 $\pm$ 0.29 <sup>c</sup>	5.01 $\pm$ 0.41 <sup>ab</sup>	0.68 $\pm$ 0.05 <sup>b</sup>	0.06 $\pm$ 0.01	0.22 $\pm$ 0.07	35.80 $\pm$ 1.24 <sup>b</sup>
<b>Vaccinated Propolis administered (Challenged GIV)</b>	13.98 $\pm$ 0.09 <sup>a</sup>	6.32 $\pm$ 0.14 <sup>a</sup>	6.38 $\pm$ 0.13 <sup>a</sup>	0.98 $\pm$ 0.09 <sup>a</sup>	0.08 $\pm$ 0.01	0.21 $\pm$ 0.06	60.80 $\pm$ 1.74 <sup>a</sup>

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a

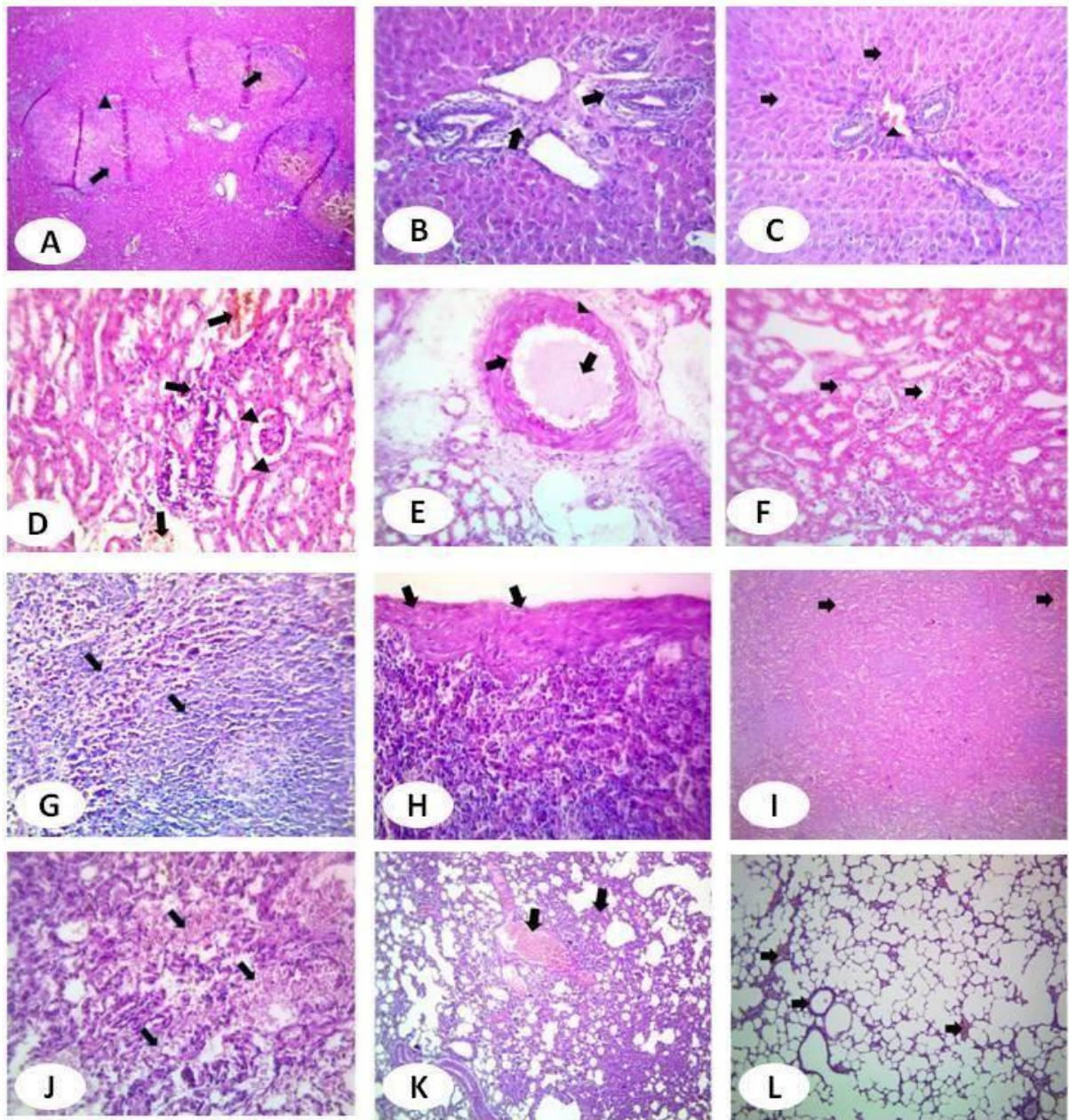
**Table 3:** Clinicobiochemical results (mean values  $\pm$  SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2<sup>nd</sup> and 6<sup>th</sup> weeks of the experiment

	ALT U/l	AST U/l	ALP U/l	T. bilirubin mg%	D. bilirubin mg%	In. bilirubin mg%	Urea mg/dl	Creatinine mg/dl	NO $\mu\text{mol/l}$
<b>2<sup>nd</sup> week</b>									
<b>Control (GI)</b>	16.18 $\pm$ 0.37 <sup>b</sup>	15.56 $\pm$ 0.41 <sup>b</sup>	41.07 $\pm$ 2.08 <sup>ab</sup>	1.00 $\pm$ 0.04 <sup>b</sup>	0.22 $\pm$ 0.03 <sup>b</sup>	0.77 $\pm$ 0.04 <sup>b</sup>	33.49 $\pm$ 1.19	0.94 $\pm$ 0.05 <sup>a</sup>	0.66 $\pm$ 0.11 <sup>b</sup>
<b>Non-vaccinated Challenged (GII)</b>	16.50 $\pm$ 0.39 <sup>b</sup>	15.20 $\pm$ 0.41 <sup>b</sup>	40.12 $\pm$ 1.68 <sup>ab</sup>	0.94 $\pm$ 0.06 <sup>b</sup>	0.24 $\pm$ 0.02 <sup>b</sup>	0.71 $\pm$ 0.07 <sup>b</sup>	34.74 $\pm$ 1.05	0.96 $\pm$ 0.05 <sup>a</sup>	0.70 $\pm$ 0.12 <sup>ab</sup>
<b>Vaccinated Challenged (GIII)</b>	18.32 $\pm$ 0.85 <sup>a</sup>	19.58 $\pm$ 0.38 <sup>a</sup>	44.82 $\pm$ 1.59 <sup>a</sup>	1.36 $\pm$ 0.06 <sup>a</sup>	0.25 $\pm$ 0.02 <sup>ab</sup>	1.10 $\pm$ 0.08 <sup>a</sup>	33.79 $\pm$ 1.33	0.98 $\pm$ 0.03 <sup>a</sup>	0.78 $\pm$ 0.07 <sup>a</sup>
<b>Vaccinated Propolis administered (Challenged GIV)</b>	16.30 $\pm$ 0.24 <sup>b</sup>	15.72 $\pm$ 0.73 <sup>b</sup>	37.67 $\pm$ 1.33 <sup>b</sup>	0.99 $\pm$ 0.03 <sup>b</sup>	0.21 $\pm$ 0.01 <sup>b</sup>	0.78 $\pm$ 0.03 <sup>b</sup>	32.83 $\pm$ 0.84	0.65 $\pm$ 0.04 <sup>b</sup>	0.63 $\pm$ 0.08 <sup>b</sup>
<b>6<sup>th</sup> week</b>									
<b>Control (GI)</b>	14.86 $\pm$ 0.44 <sup>c</sup>	13.64 $\pm$ 0.49 <sup>c</sup>	39.70 $\pm$ 2.65 <sup>c</sup>	0.95 $\pm$ 0.04 <sup>d</sup>	0.30 $\pm$ 0.02 <sup>b</sup>	0.65 $\pm$ 0.05 <sup>c</sup>	34.14 $\pm$ 1.54 <sup>b</sup>	0.95 $\pm$ 0.07 <sup>b</sup>	0.58 $\pm$ 0.08 <sup>c</sup>
<b>Non-vaccinated Challenged (GII)</b>	32.03 $\pm$ 1.08 <sup>a</sup>	27.57 $\pm$ 1.19 <sup>a</sup>	55.45 $\pm$ 2.03 <sup>a</sup>	2.05 $\pm$ 0.09 <sup>a</sup>	0.43 $\pm$ 0.03 <sup>a</sup>	1.62 $\pm$ 0.09 <sup>a</sup>	48.65 $\pm$ 1.21 <sup>a</sup>	1.57 $\pm$ 0.05 <sup>a</sup>	2.98 $\pm$ 0.15 <sup>a</sup>
<b>Vaccinated Challenged (GIII)</b>	24.49 $\pm$ 0.53 <sup>b</sup>	22.37 $\pm$ 1.04 <sup>b</sup>	44.31 $\pm$ 1.21 <sup>b</sup>	1.61 $\pm$ 0.06 <sup>b</sup>	0.29 $\pm$ 0.01 <sup>b</sup>	1.32 $\pm$ 0.05 <sup>b</sup>	34.83 $\pm$ 1.05 <sup>b</sup>	0.98 $\pm$ 0.05 <sup>b</sup>	2.06 $\pm$ 0.05 <sup>b</sup>
<b>Vaccinated Propolis administered (Challenged GIV)</b>	13.78 $\pm$ 0.45 <sup>c</sup>	13.56 $\pm$ 0.33 <sup>c</sup>	41.57 $\pm$ 0.85 <sup>bc</sup>	1.03 $\pm$ 0.01 <sup>cd</sup>	0.22 $\pm$ 0.01 <sup>c</sup>	0.81 $\pm$ 0.01 <sup>c</sup>	22.00 $\pm$ 0.95 <sup>c</sup>	0.60 $\pm$ 0.03 <sup>c</sup>	0.58 $\pm$ 0.09 <sup>c</sup>

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a



**Figure 1:** Representative photos showing; mild phagocytic activity in non vaccinated and non challenged control group (I) (a), non-vaccinated challenged group (II) (b), moderate in vaccinated challenged group (III) (c,d) and high in vaccinated propolis administered group (IV) (e,f), arrows indicate phagocytes with engulfed yeast cells



**Figure 2:** Photomicrograph of H&E-stained tissue sections: Liver (A,B&C) at X100; (A): Multiple focal areas of coagulative necrosis (arrows) surrounded by line of demarcation (arrowhead) (non-vaccinated challenged group II), (B): Mononuclear cell infiltration in the portal areas (arrows) (vaccinated challenged group III), (C): Normal hepatocytes (arrows) with mild portal congestion (arrowhead) (vaccinated propolis administered group IV). Kidneys (D,E&F) at X400; (D): Vascular congestion, mononuclear cell infiltration (arrows) with widening of the glomerular space (arrowheads) (non-vaccinated challenged group II), (E): Vascular congestion, with perivascular edema (arrows) and mononuclear cell infiltration (arrowhead) (vaccinated challenged group III), (F): Almost normal renal epithelium except for very mild vacuolations (arrows) (vaccinated propolis administered group IV). Spleen (G,H at X400&I at X100); (G): Marked polymorphonuclear cell infiltration (arrows) (non-vaccinated challenged group II), (H): Marked thickening of the splenic capsule (arrows) (vaccinated challenged group III), (I): Almost normal red and white pulps except for mild hemosiderosis (arrows) (vaccinated propolis administered group IV). Lung (J at X400, K&L at X100); (J): Extensive hemorrhage (presence of large numbers of extravasated RBCs in the alveolar lumens)(arrows) (non-vaccinated challenged group II), (K): Pneumonic area (represented by marked vascular congestion, and leukocytic infiltration) (arrows) (vaccinated challenged group III), (L): Almost normal pulmonary tissue except for a focus having mild congestion and few mononuclear cell infiltration (arrows) (vaccinated propolis administered group IV)

## Discussion

Propolis is a resinous hive product gathered by bumble bees from different plant sources. It has various biological and pharmacological properties, for example, antimicrobial, anti-inflammatory and immunomodulatory (28,29). The propolis extract may act as an assistant adjuvant substance, potentiating the humoral reaction activated by the antigen (11). Propolis flavonoids are adjuvant with immune enhancement properties, for example, improving the differentiation of T, B lymphocyte cell and expanding neutralizing antibody titers (30).

By evaluation of erythrogram in the present study, non-vaccinated (group II), after 6<sup>th</sup> week of the experiment showed a macrocytic hypochromic anemia and thrombocytopenia, that may be attributed to hemorrhage induced by *P. multocida* (31-33). Our results were confirmed by the histopathological findings in different organs from rabbits in group (II), where kidneys and lungs showed vascular congestion and extensive hemorrhage with presence of large numbers of extravasated RBCs in the alveolar lumens respectively.

Group (III) showed normocytic normochromic anemia at the end of the 2<sup>nd</sup> week of the experiment. This anemia may be due to the adverse effect of formalized killed *P. multocida* vaccine on erythropoiesis process through its effect on erythrocyte precursor cells in the bone marrow (34). However, these parameters returned to normal values of the control at the end of 6<sup>th</sup> week of the experiment. This may be attributed to the transient adverse effect of formalized killed *P. multocida* vaccine on erythropoiesis process. These results agreed with Jasprica et al. (35) and Nassar et al. (36).

Group (IV) did not show any significant changes in erythrocytic parameters during all the experimental periods. This could be attributed to protective effect of polyphenolics, components of propolis, on the red blood cell membrane (37), together with a possibility that RBC act as a natural flavonoid reservoir (38). There is a considerable and reliable evidence for antioxidant activity of propolis in vitro

(39,40). Flavonoids and phenolic acids under specific conditions in vitro can act as antioxidant preventing the undesired toxic impacts of free radicals (41).

Regarding the platelets count, groups (III and IV) did not show any significant changes in platelets count during all experimental periods when compared with control group; that suggests the positive effect of propolis and killed *P. multocida* vaccine on the number of platelets. Also, it suggests the effective role of combined propolis in elevating myeloid and megakaryocytic types of colony forming units (CFUs) of hematopoietic tissue of treated animals. This result agreed with Oršolic' and Bašić' (42).

Regarding leukogram, there was a leukocytosis with a highly significant neutrophilia at the end of 6<sup>th</sup> week in group (II) rabbits after challenging with virulent *P. multocida*. This may probably due to the severe inflammatory response induced by *P. multocida* as concluded by Wu et al. (43). Also, Galdiero et al. (44) said that the porins, main polypeptide of the outer membrane of *P. multocida*, affect various biological functions of cells involved in the immune response as well as in inflammation especially neutrophils. On the other hand, group III did not show significant changes in neutrophilic count along all the experimental periods; this may be attributed to the weak neutrophilic stimulation by the killed vaccine. This result agreed with Cho et al. (45). While, this group showed a significant increase in total leukocytic count only at the end of 2<sup>nd</sup> week of the experiment, mainly due to rise in lymphocytes that suggests the immune enhancing effect of formalized killed vaccine on the lymphocytes. In contrast, the total leukocytic count in this group return to normal values of the control at the end of 6<sup>th</sup> weeks post-treatment; this may be attributed to that inactivated vaccine enhance mainly humoral immune response for short period (46,47).

Group (IV) showed leukocytosis mainly due to rise in neutrophilic, lymphocytic and monocytic counts. This rise presents in propolis treated rabbits suggesting the immunomo-

dulatory effect of propolis alone or its enhancing effect to immunity when used as adjuvant with killed vaccine (48,49). Propolis can act as an immune stimulant as well as increase the total leukocytes value (50). Also, this rise in WBCs may be attributed to stimulation of multiplication of leukocyte precursors from pluripotent stem cells caused by propolis (42).

Our findings agreed with Orsolić and Basic (51,42) who reported that, water-soluble derivative of propolis (WSDP) given to mice caused a significant elevation of leucocytes in peripheral blood.

Concerning the phagocytic activity, group (IV) recorded the highest significant increase in the phagocytic activity followed by group (III) compared with other groups (I and II). This result may be discussed due to the immunomodulatory effect of propolis and its components (52). Also, Possamai et al. (53) found that mononuclear phagocytes exposed to propolis adsorbed onto polyethelene glycol (PEG) microspheres showed high levels of superoxide release, phagocytosis, and microbicidal activity.

Propolis flavonoids were reported as adjuvants, that have an immuno stimulation action that accelerate the transformation of T, B lymphocyte cell and augmenting neutralizing antibody titers (30). Group (III) showed a significant increase in the phagocytic activity compared with control, but this rise was not as high as that of the propolis treated rabbits. This result may be attributed to the effect of killed vaccine on induction of only humoral immune response without great effect on cell mediated immunity (45,54).

Regarding the biochemical analysis for evaluation of liver functions, group (II) at the end of 6<sup>th</sup> week of the experiment showed a highly significant rise in serum level of ALT, AST and ALP that suggests the damaging effect of virulent *P. multocida* on the liver. Similar results previously obtained by Nassar et al. (15). These results disagreed with Thurston et al. (55) who found that rats injected with a sub lethal single subcutaneous dose (0.2 microgram/kg of body weight) of *P. multocida* D toxin showed no significant changes in serum

level of these enzymes. This difference with our work may be due to difference in bacterial dose, duration of challenge or physiological difference in the animal species. Group III showed a significant increase in ALT and AST level and total and indirect bilirubin at the end of the 2<sup>nd</sup> and 4<sup>th</sup> weeks. This could be interpreted due to mild hepatic irritating effect induced by formalized killed *P. multocida* vaccine injection. The same results were previously recorded by Nassar et al. (15). On the other hand, Group IV showed non significant rise in ALT, AST and ALP level and total, direct and indirect bilirubin when compared with the control (group.I) that could be attributed to hepato-protective and improving effect of propolis on liver functions (56).

Our results were confirmed by histopathological changes in group (II), where liver showed multiple focal areas of coagulative necrosis. While, the liver of vaccinated rabbits in groups III and IV have less severe lesion represented by mononuclear cell infiltration in the portal areas and normal hepatocytes with mild portal congestion respectively. These results were in consistent with Mathy et al. (57) and Nassar et al. (37).

Serum urea and creatinine levels in bacterial challenged rabbits (group II) revealed a highly significant rise. In contrast, formalized killed vaccinated rabbits either alone (group III) or with propolis (group IV) showed non significant changes in serum urea and creatinine levels in comparison with control (group I) along the experimental period. These results proved the renal damaging effect of virulent *P. multocida* which can be prevented by administration of killed vaccine alone or in combination with propolis.

Our obtained results were established by the histopathological findings in group (II), where kidneys showed vascular congestion, mononuclear cell infiltration with widening of the glomerular space. While, the kidneys of vaccinated rabbits (group III) have slight vacuolation in renal epithelium. These results confirmed the adverse effect of virulent *P. multocida* on renal histology which can be

prevented by administration of killed vaccine alone or in combination with propolis.

## Conclusion

As an evident from the current study, it could be concluded that the use of propolis improved the immune protection of rabbits against pasteurellosis than using the vaccine alone.

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## Conflict of interest

The authors declare no conflicts of interest.

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