

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

Mohamed A. Hashem, Essam A. Mahmoud, Mohamed F.M. Farag*

Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: farag_cell@yahoo.com

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 2nd and 6th 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*

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* (9×10^9 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 5×10^9 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 5×10^9 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

5th 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 2nd and 6th weeks of the experiment. They were divided into three parts. The 1st part (1ml rabbit blood) was placed in clean Wasserman tubes containing disodium salts of EDTA for hematological examination. The 2nd part (5ml rabbit blood) was placed in a chemical free test tube without anticoagulant to separate a clear serum for biochemical analysis (16). The 3rd 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 6th 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 Research, 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% CO₂ 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 6th 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 ± 0.07), Hb (11.38 ± 0.10), PCV (35.71 ± 0.90), MCV (62.89 ± 1.44), MCHC (31.94 ± 0.67), and platelets (456.8 ± 13.58) at the end of the 2nd week from starting the experiment. Meanwhile, at the end of the 6th 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 2nd week with non-significant changes at the counts of platelets (448.8 ± 36.12) but at the end of the 6th 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 2nd 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 ± 0.42) including neutrophils (3.84 ± 0.28), lymphocytes (4.21 ± 0.28), monocytes (0.77 ± 0.09), eosinophils (0.08 ± 0.02) and basophils (0.24 ± 0.09) counts. Meanwhile, at the end of

the 6th week, group (II) showed a highly significant increase in total leukocytic (13.35 ± 0.20) and neutrophilic (8.01 ± 0.25) count with non-significant changes at the other leukocytes. On the other side, group (III) revealed leukocytosis (11.63 ± 0.39) and lymphocytosis (5.99 ± 0.26) at the end of the 2nd week with non significant change in leukogram variables at the end of 6th 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 ± 1.5 and 23.8 ± 1.24) than the control group (I) along experimental periods (Figure 1a), elevated in group (III) (35.8 ± 1.24) (Figure 1c,d) and highly increased in group (IV) (60.20 ± 1.59 and 60.8 ± 1.74) (Figure 1e,f).

Biochemical results

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

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

with control group (I), but at the 6th week, it showed a decrease in urea (22.0 ± 0.95), creatinine (0.6 ± 0.03) and direct bilirubin (0.22 ± 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 2nd and 6th weeks of the experiment

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

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 2nd and 6th weeks of the experiment

	TLC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Phagocytic %
2nd week							
Control (GI)	9.16 $\pm$ 0.74 ^c	4.11 $\pm$ 0.15 ^{bc}	3.98 $\pm$ 0.47 ^b	0.78 $\pm$ 0.28 ^b	0.06 $\pm$.01	0.23 $\pm$ 0.06	26.20 $\pm$ 1.39 ^c
Non-vaccinated Challenged (GII)	8.84 $\pm$ 0.42 ^c	3.84 $\pm$ 0.28 ^c	4.21 $\pm$ 0.28 ^b	.077 $\pm$ 0.09 ^b	0.08 $\pm$ 0.02	0.24 $\pm$ 0.09	25.60 $\pm$ 1.50 ^c
Vaccinated Challenged (GIII)	11.63 $\pm$ 0.39 ^b	4.53 $\pm$ 0.18 ^b	5.99 $\pm$ 0.26 ^a	0.81 $\pm$ 0.07 ^b	0.09 $\pm$ 0.01	0.20 $\pm$ 0.08	35.80 $\pm$ 1.24 ^b
Vaccinated Propolis administered (Challenged GIV)	14.41 $\pm$ 0.38 ^a	6.44 $\pm$ 0.17 ^a	6.03 $\pm$ 0.26 ^a	1.63 $\pm$ 0.18 ^a	0.08 $\pm$ 0.02	0.24 $\pm$ 0.06	60.20 $\pm$ 1.59 ^a
6th week							
Control (GI)	9.29 $\pm$ 0.41 ^b	4.40 $\pm$ 0.19 ^c	4.03 $\pm$ 0.60 ^b	0.59 $\pm$ 0.04 ^{bc}	0.06 $\pm$ 0.01	0.21 $\pm$ 0.08	25.60 $\pm$ 1.50 ^c
Non-vaccinated Challenged (GII)	13.35 $\pm$ 0.20 ^a	8.01 $\pm$ 0.25 ^a	4.50 $\pm$ 0.15 ^b	0.63 $\pm$ 0.04 ^b	0.05 $\pm$ 0.00	.020 $\pm$ 0.05	23.80 $\pm$ 1.24 ^c
Vaccinated Challenged (GIII)	10.21 $\pm$ 0.71 ^b	4.24 $\pm$ 0.29 ^c	5.01 $\pm$ 0.41 ^{ab}	0.68 $\pm$ 0.05 ^b	0.06 $\pm$ 0.01	0.22 $\pm$ 0.07	35.80 $\pm$ 1.24 ^b
Vaccinated Propolis administered (Challenged GIV)	13.98 $\pm$ 0.09 ^a	6.32 $\pm$ 0.14 ^a	6.38 $\pm$ 0.13 ^a	0.98 $\pm$ 0.09 ^a	0.08 $\pm$ 0.01	0.21 $\pm$ 0.06	60.80 $\pm$ 1.74 ^a

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 2nd and 6th 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 $\mu\text{mol/l}$
2nd week									
Control (GI)	16.18 $\pm$ 0.37 ^b	15.56 $\pm$ 0.41 ^b	41.07 $\pm$ 2.08 ^{ab}	1.00 $\pm$ 0.04 ^b	0.22 $\pm$ 0.03 ^b	0.77 $\pm$ 0.04 ^b	33.49 $\pm$ 1.19	0.94 $\pm$ 0.05 ^a	0.66 $\pm$ 0.11 ^b
Non-vaccinated Challenged (GII)	16.50 $\pm$ 0.39 ^b	15.20 $\pm$ 0.41 ^b	40.12 $\pm$ 1.68 ^{ab}	0.94 $\pm$ 0.06 ^b	0.24 $\pm$ 0.02 ^b	0.71 $\pm$ 0.07 ^b	34.74 $\pm$ 1.05	0.96 $\pm$ 0.05 ^a	0.70 $\pm$ 0.12 ^{ab}
Vaccinated Challenged (GIII)	18.32 $\pm$ 0.85 ^a	19.58 $\pm$ 0.38 ^a	44.82 $\pm$ 1.59 ^a	1.36 $\pm$ 0.06 ^a	0.25 $\pm$ 0.02 ^{ab}	1.10 $\pm$ 0.08 ^a	33.79 $\pm$ 1.33	0.98 $\pm$ 0.03 ^a	0.78 $\pm$ 0.07 ^a
Vaccinated Propolis administered (Challenged GIV)	16.30 $\pm$ 0.24 ^b	15.72 $\pm$ 0.73 ^b	37.67 $\pm$ 1.33 ^b	0.99 $\pm$ 0.03 ^b	0.21 $\pm$ 0.01 ^b	0.78 $\pm$ 0.03 ^b	32.83 $\pm$ 0.84	0.65 $\pm$ 0.04 ^b	0.63 $\pm$ 0.08 ^b
6th week									
Control (GI)	14.86 $\pm$ 0.44 ^c	13.64 $\pm$ 0.49 ^c	39.70 $\pm$ 2.65 ^c	0.95 $\pm$ 0.04 ^d	0.30 $\pm$ 0.02 ^b	0.65 $\pm$ 0.05 ^c	34.14 $\pm$ 1.54 ^b	0.95 $\pm$ 0.07 ^b	0.58 $\pm$ 0.08 ^c
Non-vaccinated Challenged (GII)	32.03 $\pm$ 1.08 ^a	27.57 $\pm$ 1.19 ^a	55.45 $\pm$ 2.03 ^a	2.05 $\pm$ 0.09 ^a	0.43 $\pm$ 0.03 ^a	1.62 $\pm$ 0.09 ^a	48.65 $\pm$ 1.21 ^a	1.57 $\pm$ 0.05 ^a	2.98 $\pm$ 0.15 ^a
Vaccinated Challenged (GIII)	24.49 $\pm$ 0.53 ^b	22.37 $\pm$ 1.04 ^b	44.31 $\pm$ 1.21 ^b	1.61 $\pm$ 0.06 ^b	0.29 $\pm$ 0.01 ^b	1.32 $\pm$ 0.05 ^b	34.83 $\pm$ 1.05 ^b	0.98 $\pm$ 0.05 ^b	2.06 $\pm$ 0.05 ^b
Vaccinated Propolis administered (Challenged GIV)	13.78 $\pm$ 0.45 ^c	13.56 $\pm$ 0.33 ^c	41.57 $\pm$ 0.85 ^{bc}	1.03 $\pm$ 0.01 ^{cd}	0.22 $\pm$ 0.01 ^c	0.81 $\pm$ 0.01 ^c	22.00 $\pm$ 0.95 ^c	0.60 $\pm$ 0.03 ^c	0.58 $\pm$ 0.09 ^c

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

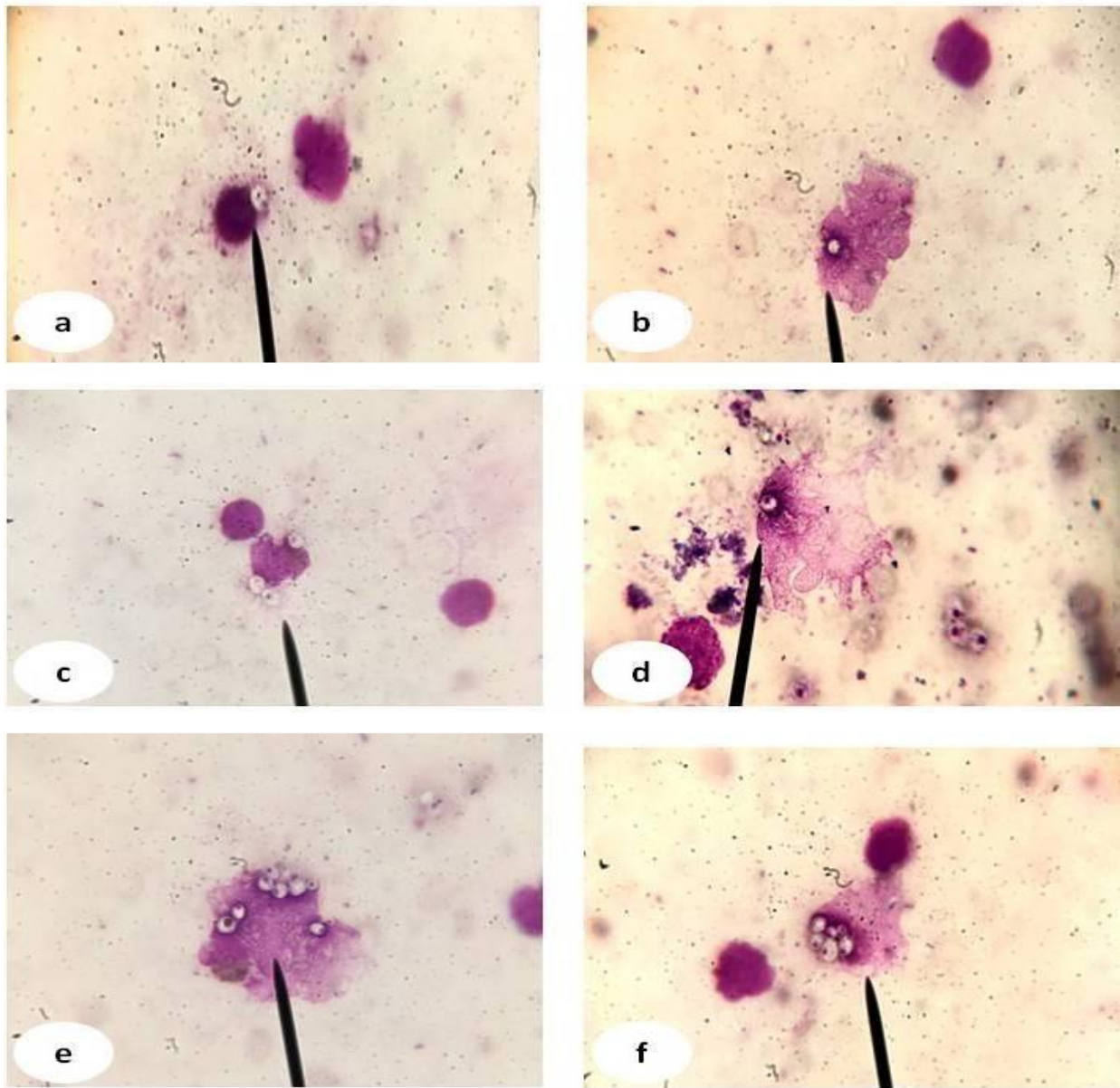


Figure 1: Representative photos showing; mild phagocytic activity in non vaccinated and non challenged control group (I) (a), non-vaccinated challenged group (II) (b), moderate in vaccinated challenged group (III) (c,d) and high in vaccinated propolis administered group (IV) (e,f), arrows indicate phagocytes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

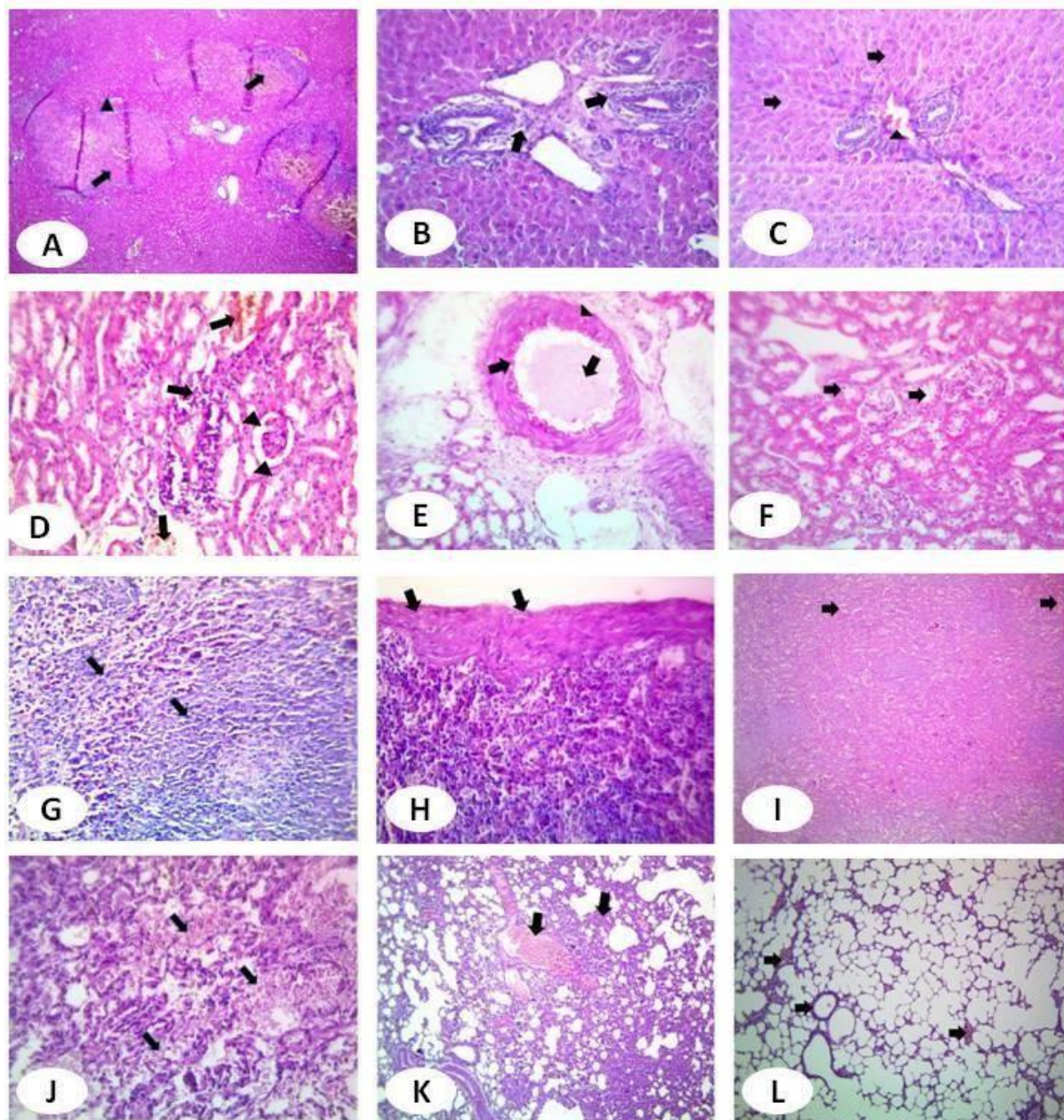


Figure 2: Photomicrograph of H&E-stained tissue sections: Liver (A,B&C) at X100; (A): Multiple focal areas of coagulative necrosis (arrows) surrounded by line of demarcation (arrowhead) (non-vaccinated challenged group II), (B): Mononuclear cell infiltration in the portal areas (arrows) (vaccinated challenged 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 6th 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 2nd 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 6th 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 6th 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 2nd 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 6th 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 immuno-modulatory 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 6th 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 2nd and 4th 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.

Acknowledgment

We thanks and appreciates Dr. Mohamed Metwally M., assistant professor of pathology, Department of Pathology, Faculty of veterinary medicine, Zagazig University, Egypt for his help in performing the histopathology.

Conflict of interest

The authors declare no conflicts of interest.

References

1. Zimmerman T., Deeb B., and DiGiacomo R. Polypeptides associated with *Pasteurella multocida* infection in rabbits. Am J of Vet Res 1992; 53: 1108–12.
2. Gaertner D. Comparison of penicillin and gentamycin for treatment of pasteurellosis in rabbits. Lab Anim Sci 1991; 41: 78-80.
3. Okerman L and Spanoghe L. Protective effects of inactivated *pasteurella* vaccines in specific pathogen free rabbits. Comparative Immunology, Microbiology and Infectious Diseases 1981; 4: 223–8.
4. Okerman L and Spanoghe L. Immunity induced in mice by *Past. multocida* strain isolated from rabbits. Zentrablatt fur Veterinarmedizin 1980; 37 B (9/10): 759–63.
5. Percy D, Prescott J and Bhasin J. Inactivated bacterial antigen were complemented with adjuvants in order to increase the immunogenicity of the vaccines. J Complementary Med 1985; 46: 227–9.
6. Baljer G, Charcherr S and Mayr A. Efficacy and harmlessness of inactivated *Past. multocida* vaccines on subcutaneous, oral and intranasal application in mice. Zentralatt fur veterinarmedizin 1982; 27: 275–83.
7. Shawky T. The immuno-potentiating Impact of zinc and vit.E in buffalo calves. Master thesis, Cairo University, Giza, Egypt 1996.
8. Amal F. Pharmacological studies on *Nigella sativa* L on chickens genotoxic changes during LPS induced endotoxemia. Phytomedicine 1997; 20: 632–9.
9. Abd El-Fattah A, Mohamed E, Mohamed E and Ramadan G. Effect of thymus extract on immunologic reactivity of chicken vaccinated with infectious bursal disease virus. Journal of Veterinary Medical Science 1999; 61:811–7.
10. Boutabet K, Kebba W, Alyane M and Lahouel M. Polyphenolic fraction of Algerian propolis protects rat kidney against acute oxidative stress induced by doxorubicin. Indian J. of Nephrology 2011; 21(2):101–6.
11. Fischer G, Cleff M, Dummer L, Paulino N, Paulino A, Vilela C, Campos F, Storch T, Vargas D, Huber S and Vidor T. Adjuvant effect of green propolis on humoral immune response of bovines immunized with bovine herpesvirus type 5. Veterinary Immunology and Immunopathology 2007; 116: 79–84.
12. El-Akabawy L, Zayan K, Tanatawy A and Omer R. Anticoccidial efficacy of propolis and toltrazuril against *Eimeria stiedai* in New Zealand white rabbits .Zag.Vet.J. 2004; 32:129–45.
13. Cruickshank R, Duguid J, Marmion B and Swain R. Medical Microbiology, vol. 2, 12th edition. Churchill Livingstone, Edinburgh, UK 1975; 96–150.
14. Osama A. Evaluation of the immune response against *Past. multocida* vaccines. Master thesis (Microbiology), Faculty of Vet. Med., Zagazig University 1997.
15. Nassar S, Mohamed A, Soufy H, Nasr S, and Mahran K. Immunostimulant Effect of Egyptian Propolis in Rabbits. The Scientific World Journal 2012; 1–9.
16. Coles E. Veterinary Clinical Pathology.4th edition. W.B. Saunders Company. Philadelphia 1986.
17. Reitman S and Frankel S. Colorimetric method for determination of serum transaminases activities. Am J Clin Path 1957; 28: 56-63.
18. Tietz N. Fundamentals of Clinical Pathology. W.B Saunders Company. Philadelphia 1976; 602–9.
19. Fuehr J. Bilirubin determination of the serum according to the method of L. Jendrassik, R.A. Cleghorn and P. Grof. Med Monatsschr 1964; 18:183–4.
20. Henry T. Clinical Chemistry .Principles and Techniques, 2nd edition. Harper and Row Publishers. New York 1974.
21. Fawcett J and Scott J. A rapid and precise method for the determination of urea. Journal of Clinical Pathology 1960; 13:156.

22. Montgomery H and Dymock J. The determination of nitrite in water. *Analyst* 1961; 86: 414–6.
23. Waterstrat P, Ainsworth A and Capley G. Use of a discontinuous Percoll gradient technique for the separation of channel catfish peripheral blood leukocytes. *J Fish Dis* 1988; 11: 289–94.
24. Ourth D and Chung K. Purification of antimicrobial factor from granules of channel catfish peripheral blood leucocytes. *Biochemical and Biophysical Research Communications* 2004; 313: 28–36.
25. Ainsworth A and Chen D. Differences in the phagocytosis of four bacteria by channel catfish neutrophils. *Dev. Comp. Immunol.* 1990; 14: 201–9.
26. Bancroft J, Stevens A and Turner D. *Theory and Practice of Histological Technique*, 4th edition. Churchill, Livingstone, New York, London, Sanfrancisco, Tokyo 1996.
27. SPSS (2001): SPSS/PC + (2001), for the PC /XT. SPSS INC
28. Pari L and Gnanasoundari M. Influence of naringenin on oxytetracycline mediated oxidative damage in rat liver. *Basic and Clinical Pharmacology and Toxicology* 2006; 98: 456–61.
29. Orsatti C, Missima F, Pagliarone A and Sforzin J. Th1/Th2 cytokines' expression and production by propolistreated mice. *Journal of Ethnopharmacology* 2010; 129: 314–8.
30. Wang D, Hu Y, Sun J, Kong X, Zhang B, and Liu J. Comparative study on adjuvanticity of compound Chinese herbal medicinal ingredients. *Vaccine* 2005; 23: 3704–8.
31. Manche H and Toll H. Pulmonary cavitation and massive hemorrhage caused by *Pasteurella multocida*. *The new England Journal of Medicine* 1964; 271:491–4.
32. Jaglic Z, Jeklova E, Christensen H, Leva L, Register K, Kummer V, Kucerova Z, Faldyna M, Maskova J and Nedbalcova K. Host response in rabbits to infection with *Pasteurella multocida* serogroup F strains originating from fowl cholera. *Can J Vet Res* 2011; 75:200–8.
33. Chung E, Abdullah F, Marza A, Saleh W, Ibrahim H, Abba Y, Zamri-Saad M, Haron A, Saharee A, Lila M and Norsidin M. Clinico-pathology and hematobiochemistry responses in buffaloes infected with *Pasteurella multocida* type B:2 immunogen outer membrane protein. *Microbial Pathogenesis* 2017; 11.
34. Kleinrok Z, Borzecki Z, Scheller S and Matuga W. Biological properties and clinical application of propolis. X. Preliminary pharmacological evaluation of ethanol extract of propolis (EEP). *Arzneimittel-Forschung* 1987; 28 (2): 291–2.
35. Jasprica I, Mornar A and Debeljak Z. *In vivo* study of propolis supplementation effects on antioxidative status and red blood cells. *J Ethnopharmacology* 2007; 110 (3): 548–54.
36. Nassar S, Mohamed A, Soufy H and Nasr S. Protective Effect of Egyptian Propolis against Rabbit Pasteurellosis. *BioMed. Research International* 2013; 163724: 1–9.
37. Youdim K, Shukitt-Hale B, MacKinnon S, Kalt W and Joseph J. Polyphenolics enhance red blood cell resistance to oxidative stress: in vitro and in vivo. *Biochimica et Biophysica Acta* 2000; 1523: 117–22.
38. Fiorani M, Accorsi A and Cantoni O. Human red blood cells as a natural flavonoid reservoir. *Free Radical Research* 2003; 37: 1311–38.
39. Kumazawa S, Hamasaka T and Nakayama T. Antioxidant activity of propolis of various geographic origins. *Food Chemistry* 2004; 84: 329–39.
40. Russo A, Cardile V, Sanchez F, Troncoso N, Vanella A and Garabino J. Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines. *Life Sciences* 2004; 76: 545–58.
41. Galati G and O'Brien P. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine* 2004; 37: 287–303.
42. Orsolić N and Basic I. Antitumor, hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP). *Biomed. Pharmacother.* 2005; 59: 561–70.
43. Wu Q, Yub L, Qiu J, Shen B, DiWangb, Soromou L and Fenga H. Linalool attenuates lung inflammation induced by *Pasteurella multocida* via activating Nrf-2 signaling pathway *International. Immunopharmacology* 2014; 5:1567–9.
44. Galdiero M, Palomba E, De L, Vitiello M and Pagnini P. Effects of the major *Pasteurella multocida* porin on bovine neutrophils. *Am J Vet Res* 1998; 59:1270–4.
45. Cho S, Park J, Kim J and Yoon Y. Studies on the development of combined vaccine for control of snuffles (*Past. multocida*, *Bordetella bronchiseptica* infections). *Research Report of Rural Development Administration Veterinary* 1989; 31: 29–37.

46. Borkowska O, Kedrak A and Truszczyński M. *Pasteurella multocida* serum level in rabbits vaccinated against pasteurellosis. Bulletin of the Veterinary Institute in Pulawy 1996; 40: 97–104.
47. Borkowska O, Kedrak A and Truszczyński M. Application of the ELISA for determination of anti *Pasteurella multocida* IgG in sera of rabbit vaccinated against pasteurellosis under field condition. Bulletin of the Veterinary Institute in Pulawy 1997; (41): 17–24.
48. Dimov V, Ivanovska N, Bankova V, Popov S, Manonlova N and Nikolov N. Immuno-modulatory action of propolis Influence on anti-infections protection and macrophage function. Apidologie 1991; 22: 155–62.
49. Moriyasu J, Arai S, Motoda R, and Kurimoto M. In vitro activation of mouse macrophage by propolis extract powder. Biotherapy 1994; 8: 364–8.
50. Dimov V., Ivanovska N., Bankova V., Popov S., Manonlova N. and Nikolov N. Immuno-modulatory action of propolis. Influence on anti-infections protection and macrophage function. Apidologie 1991; 22: 155–62.
51. Orsolic N and Basic I. Immuno-modulation by water soluble derivatives of propolis: a factor of anti-tumour reactivity. Journal of Ethnopharmacology 2003; 84: 265–73.
52. Bratter C, Tregel M, Liebenthal C and Volk H. Prophylactic effectiveness of propolis for immunostimulation: a clinical pilot study. Forsch Komplementarmed 1999; 6: 256–60.
53. Possamai M, Honorio-França A, Reinaque A, França E and Souza Souto P: Brazilian Propolis. A Natural Product That Improved the Fungicidal Activity by Blood Phagocytes. BioMed. Research International 2013; 54: 1018–27.
54. Jarvinen L, Hogenesch H, Suckow M and Bowersock T. Induction of protective immunity in rabbits by coadministration of inactivated *Pasteurella multocida* toxin and potassium thiocyanate extract. Infection and Immunity 1998; 66: 3788–95.
55. Thurston J, Cheville N, Rimler R and Sacks J. Serum complement activity and serum enzymes in rats after a subcutaneous injection of toxin prepared from *Pasteurella multocida* type D. Vet Immunol Immunopathol 1989; 23 (3-4): 385–8.
56. Morsy A, Abdalla A, Soltan Y, Sallam S, El-Azrak K, Louvandini H and Alencar S. Effect of Brazilian red propolis administration on hematological, biochemical variables and parasitic response of Santa Inês ewes during and after flushing period. Trop. Anim. Health Prod. 2013; 45:1609–18.
57. Mathy N, Mathy J, Lee R, Walker J, Lofthouse S and Meeusen E. Pathological and immunological changes after challenge infection with *pasteurella multocida* in naïve and immunized calves. Veterinary immunology and immunopathology 2002; 85: 179–88.